THE TYPE OF PHAGOCYTIC CELL AND ITS RELATIVE PROPORTIONS IN HUMAN BONE MARROW AND SPLEEN, AS IDENTIFIED BY THE SUPRAVITAL TECHNIQUE, WITH SPECIAL REFERENCE TO PERNICIOUS ANEMIA.

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In a recent publication, Peabody and Broun (1) have reemphasized, in the light of the increasing appreciation of the rôle of phagocytosis, the relatively large number of phagocytic cells to be found in the bone marrow in pernicious anemia. Sabin, Doan, and Cunningham (2), in a survey of the phagocytic cells of the spleen, bone marrow, connective tissues, and serous exudates, by means of a supravital technique, have separated these mononuclear cells, the so called reticulo-endothelial apparatus of Aschoff, into *two* distinct strains, monocytes and clasmatocytes. The basis of the separation is fourfold, derivational, developmental, cytological, and functional. The clasmatocyte, probably of endothelial origin, and now of recognized importance in the physiological destruction of old red blood cells, is of particular interest in its possible relationship to another function attributed, at least tentatively, to endothelium (3); *viz.*, that of being the ultimate source of the red blood cells. It was with the purpose of determining the type of phagocytic cell, which is found in increased numbers in the bone marrow in pernicious anemia, that these supravital studies were made.

All cases reported were followed clinically on the Thorndike and Fourth Medical Services of the Boston City Hospital, and the diagnoses were personally confirmed. To the cooperation of the Pathological Department of the Boston City Hospital we are indebted for our postmortem material, all of which was secured within 5 hours of death, several cases within 2 hours. During the past year we have studied preparations of living cells from four cases of pernicious anemia and from six patients dying of other causes. Two cases have had biopsy exam-

inations of the bone marrow. The preparations of bone marrow and spleen were made in their own serum if a sufficient amount was present, or with the addition of warm physiological salt solution when necessary. Films of vital neutral red-Janus green were used, according to the technique and criteria described elsewhere (3). Several different preparations were always studied and from 500 to 1000 cells counted in arriving at the relative percentages of the different types.

TABLE I.

Case	1	2	3	4	5	6	7	8	9	10
Diagnosis	Pernicious anemia.				Aplastic anemia.	Coronary infarct.	Acute myeloblastic leucemia.	Nephritis.	Cirrhosis.	Lobar pneumonia.
	Bone marrow.									
				Biopsy.						
PMN. active	0.5	12.0	7.0	1.0	1.0	12.0	2.0	5.0	15.0	19.2
Myeloblasts	3.0	0.0	0.0	0.5	0.0	0.0	38.0	0.0	0.0	4.0
Myelocytes A.	0.0	1.0	0.6	1.3	2.0	0.0	35.0	1.0	2.0	4.0
" B.	48.0	19.0	29.3	18.0	9.0	10.0	9.0	10.0	10.0	10.0
" C.	30.0	50.0	44.0	25.0	34.0	70.0	6.0	60.0	40.0	50.5
PME. " C.	6.0	7.0	4.6	3.2	14.0	4.0	2.0	1.0	2.0	0.6
PMB. " C.	0.0	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.5	0.0
Lymphocytes	0.0	0.0	0.6	5.5	15.0	0.0	1.0	2.0	4.5	0.0
Monocytes	0.0	0.0	0.0	0.4	2.0	0.0	0.0	1.0	2.0	0.0
Clasmatocytes	8.0	10.0	10.0	6.0	2.0	3.0	3.0	2.0	3.5	0.3
Megacaryocytes	0.5	0.0	0.3	0.2	0.0	1.0	0.0	1.0	0.5	0.0
Normoblasts	—	—	—	22.0	21.0	—	4.0	15.0	16.0	8.4
Erythroblasts	—	—	—	14.0	0.0	—	—	2.0	4.0	3.0
Megaloblasts	4.5	0.0	3.3	2.1	0.0	0.0	0.0	0.0	0.0	0.0
	Spleen.									
Clasmatocytes	0.5	1.0	0.5	—	5	4.0	8.0	6.0	5.0	4.0

Table I summarizes the differential cell counts on the series of cases studied. It will be noted that the first four cases show a noticeably higher percentage of clasmatocytes in the bone marrow

than do the last six. The first three, all cases of fatal pernicious anemia, have strikingly similar numbers of phagocytic cells, 8 to 10 per cent. Qualitatively, these cells all showed extreme phagocytosis, many containing more than twenty ingested red blood cells, apparently freshly taken in, as they were unchanged in appearance from those outside the phagocytes.[1] In these three cases, probably the most striking feature was the very large number of ingested *nucleated* red blood cells. The peripheral blood at no time in any case showed more than a very occasional normoblast. The inference is that, in these grossly and microscopically hyperplastic marrows, the abnormally stimulated clasmatocytes were not only ingesting old, and probably functionally mature red blood cells, but immature, young forms as well, which had not yet been delivered into the general circulation. The nature of the stimulus, whether acting directly through the phagocytic cells themselves or indirectly through a hypothetical injury to the red cells, is, of course, problematic. Such an analysis and interpretation, at least, indicate the large part which these phagocytic cells may play in contributing to the anomalous situation recognized in pernicious anemia; *viz.*, that of an apparently hyperactively functioning marrow with, nevertheless, a rapidly progressive, idiopathic anemia.

In the gross, the femoral bone marrow from the cases of pernicious anemia was red throughout, as was also that of the ribs; in consistency, gelatinous and elastic, not friable or granular. It had the appearance of a marrow in which there is a stimulation of the red cell-forming elements.

Microscopically, preparations of the fresh bone marrow showed all of the cells to be viable at the time of examination, since all reacted vitally to neutral red-Janus green, and the polymorphonuclear neutrophils were actively motile. At once one was struck with the frequency of large phagocytic cells. Here and there occasional, less actively phagocytic forms were identified by their clear, round nuclei and a glassy, refractile cytoplasm, with possibly one cell inclusion.

[1] Phagocytized red blood cells undergo chemical changes after ingestion, as is indicated by a difference in reaction to supravital dyes and by observable changes in form and structure. A vacuole, in the terminology of Sabin (6), eventually marks the position of an ingested red blood cell, or cells.

But by far the majority were more than normally engorged, and contained unchanged, nucleated and non-nucleated red blood cells and intact leucocytes, together with vacuoles filled with finer, partially digested debris, and reacting with varying shades to the neutral red. Even when only one phagocytized red cell was included it was invariably found in juxtaposition to the nucleus. No clear centrosphere with a "rosette," typical of the monocyte, was observed in any cell. The mitochondrial content was sparse in all, negligible in most. All stages of maturity and activity in the clasmatocytes were seen, with from none to one to twenty cell inclusions. Some single oil immersion fields under the microscope contained as many as four engorged clasmatocytes. Every phagocytic cell, then, seen in the bone marrow from cases of pernicious anemia has been of the endothelial wandering cell type, which we designate as the *clasmatocyte*, and which, in its range of differential features, has already been described and illustrated elsewhere (2).

Occasional polymorphonuclear neutrophilic leucocytes showed a phagocytized nucleated red blood cell of the macrocyte type; a few showed single phagocytized mature microcytes. The percentages of myelocytes and their degrees of maturity, indicated in the convention A for the youngest to C for the oldest, are presented in the table. In normal bone marrow the great majority of the myelocytes are in the C, or preleucocytic, phase, making a large, readily available, potential reserve of cells, quickly convertible into mature functioning units, both regularly and on unusual demand. It is of interest, in this connection, to note that in the cases of pernicious anemia there was a partial throwback to the B level, or less readily available type of myelocyte, which may be correlated with the well known mild leucopenia with relative lymphocytosis characteristic of the disease.

Erythroblasts of all stages of maturity with a relative, limited increase in the younger forms, as contrasted with a normal marrow, were present. Megaloblasts, the earliest hemoglobinophilic primitive cells (3), were found only in the cases of pernicious anemia. It was quite obvious that the majority of the phagocytized red blood cells had been freshly ingested, and were not necessarily senile cells, for they showed the same reactions as the red cells freely floating about, and, moreover, many of them were nucleated.

Wright's stains of both dried and Zenker-fixed films corroborated the supravital findings, with the exception that most of the phagocytic clasmatocytes were destroyed, apparently through rupture of the cell membrane, so that in the fixed preparations this striking feature of the vital studies did not become so evident. After several hours in the warm box the vital preparations tended to show decreased numbers of the more heavily loaded cells. Whereas the early supravital counts averaged 8 to 10 per cent of these cells, later counts of the identical preparations and fields, without additional trauma to, or manipulation of, the cells, and the fixed preparations, similarly averaged only 3 to 5 per cent. These two observations may indicate either that the clasmatocytes are inherently fragile cells, or that the fully engorged ones are, on the digestion of their content, ready to disintegrate as a part of the process of their normal physiological function as scavengers. The latter seems more probable in the light of certain experimental observations.

Studies of the red cells with brilliant cresyl blue showed many cells with all stages and degrees of reticulum. No abnormal precipitation of the basophilic substance was detected in any instance.

In Cases 1 and 2 the spleens were slightly enlarged, firm, deep red, with the cut surfaces showing some serosanguineous exudate. In Case 3 the spleen was not enlarged. Fresh supravital studies were made as with the bone marrow. The cells reacted uniformly to the dyes with cytoplasmic bodies staining only, the criterion for a true supravital reaction. Small lymphocytes predominated. Occasional myelocytes, type C, were seen. Only a very occasional phagocytic clasmatocyte (1/2 to 1 per cent) was seen. However, those cells present were typical phagocytes, and their cell content paralleled that found in the bone marrow. Wright's stains also confirmed these observations. In contrast to the content of phagocytic cells in the spleens from these cases of pernicious anemia, the clasmatocytes in the other cases of this series ranged from 4 to 8 per cent. Necessarily any actual figures taken from differential counts from the spleen must, in the very nature of the procedure, vary widely. But, when numerous different preparations are made and in each case studied carefully, the impression of the difference in content of phagocytic cells from case to case is suggestively striking.

In Case 4 (pernicious anemia) an interesting result was obtained in the examination of the femoral bone marrow removed at biopsy.

This patient had been going down hill for a period of weeks, despite repeated transfusions at about 10 day intervals. There had been no clinical evidence of any remission in the course of the disease at the time the biopsy was decided upon. In the gross, the appearance of the bone marrow was typical of the condition. Microscopically, it presented a most interesting and suggestive picture. A definite percentage of megaloblasts was seen, but the striking feature was the large number of erythroblasts and normoblasts. The clasmatocytes, while 6 per cent in number, were very small, the majority seeming to contain debris and small vacuoles not identifiable as ingested red blood cells. Only one phagocytized nucleated red blood cell was found in many preparations. No cells with the large numbers of ingested red cells, so characteristic of the marrows from the fatal cases of pernicious anemia, were found. The interpretation from the picture presented by this marrow, and without reference to the clinical findings, would have to be that this patient was making plenty of good red cells, without undue activity on the part of the phagocytic group in destroying them; in other words, that he was in a phase of cell production exceeding cell destruction. Within 48 hours the patient began to show clinically evidences of the inauguration of a real remission, and his peripheral blood count, then and subsequently, corroborated this. 3 weeks previous to the biopsy just reported, a similar removal of bone marrow from the other femur had been made on the same patient. Vital studies were not made but the tissue was fixed and sectioned. Contrasting the stained sections from the first and second biopsy tissue, a definite impression, in the absence of actual differential counts, was gained of greater numbers of phagocytic cells with more marked phagocytosis of red cells, in the former than in the latter. A definite shift from the younger, more immature cells toward increased numbers of normoblasts was also apparent in the sections of marrow from the second biopsy. It may be said, then, that at least 48 hours preceding any clinical evidence of a remission in this case, apparently very definite changes were taking place in the bone marrow, consisting of a decrease in the usual numbers, and more particularly in the activity, of the phagocytic cells, with a decided increase in the numbers and effective developmental activity of the erythroblastic series. This simply confirms by observation that which one would suspect; *viz.*, that changes in the hemopoietic centers must precede an observable change in the clinical course of the disease. The interesting point is the apparent earlier cessation in activity of the phagocytic cells in the marrow, preceding the effective increase in red cell activity and clinical improvement.

Case 5 was one with a questionable diagnosis of pernicious anemia. At biopsy, 2 weeks before death, only fatty marrow, entirely acellular, was secured from the femur. No differential count was possible

at that time. At autopsy the same fatty condition of the marrow was found in all the bones, with the exception of a small, circumscribed area very near the epiphysis of one femur; the differential count made from that one small area is recorded. The clasmatocytes were normal in number, 2 per cent. In numerous other preparations not a single cell of either the myeloid or erythroid series could be found. The spleen showed a normal percentage (5 per cent) of clasmatocytes. In so far as the hemopoietic centers were concerned it was practically an aplastic anemia. It will be noted that the lymphocytes in this case were more numerous (15 per cent) than in any bone marrow studied, which again emphasizes the depression in activity of the myeloid centers.

The control cases, which were of non-anemic conditions, showed nothing particularly striking in their differential counts or in the development of the myeloid or erythroid series. Case 7, acute myeloblastic leucemia, shows the reversion in the myeloid series to the myeloblastic "level" of proliferation (5), with a corresponding disappearance of late myelocytes and leucocytes.

If it should be ultimately found, as it now seems possible, that the endothelium of the adult human is the *sine qua non* for both definitive red blood cells and one group of free phagocytic cells, *viz.* the clasmatocytes, we see in the disease known as pernicious anemia a vicious circle of death within one family of closely related cells. The endothelial progenitor of the essential red blood cell, under some, as yet, unknown stimulus gives rise by multiplication to unusual numbers of a type of cell with phagocytic properties, which might be designated as a first cousin of the red cell, which ingest and disintegrate the red cells, often before they have reached the stage of complete maturation.

CONCLUSION.

In general, there is a reversal of the normal in the ratio of clasmatocytes in the spleen to clasmatocytes in the bone marrow in pernicious anemia, with a marked tendency toward the phagocytosis of young, immature, nucleated red blood cells in the bone marrow. The peripheral blood picture suggests that these cells had never been in circulation. The observations made do not indicate that the spleen

takes any directly active part in an increased destruction of blood in pernicious anemia.

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EFFECT OF THYROIDECTOMY AND OF THYMECTOMY IN EXPERIMENTAL SYPHILIS OF THE RABBIT.

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In previous papers we have emphasized the importance of the integrity and balance of the system of the glands of internal secretion as one of the factors participating in the reaction of the host to the growth and fate of a transplantable neoplasm of the rabbit (1). This conception was primarily based upon well defined gross and histologic changes in a number of the endocrine glands, and it was felt that there is an essential connection between the growth of the transplanted tumor and certain members of the endocrine system, on the one hand, and the mechanism of animal defense, on the other. Both the thyroid and the thymus glands, in particular, appeared to be profoundly affected, and it was found (2) that the malignancy of the neoplasm was greatly increased in those rabbits in which the thyroid gland was completely removed either before or shortly after inoculation, while a partial thyroidectomy usually resulted in a disease of lessened malignancy. The effect of thymectomy was less clear.

In rabbits infected with *Treponema pallidum*, changes in the glands of internal secretion were also found and these in turn could be related to the mechanism of animal defense as expressed by the clinical manifestations of the disease (3). In order to test further the conception of the importance of this system of organs in the reaction of the host to disease, experiments were undertaken in which the thyroid or thymus glands of rabbits were removed prior to inoculation with *Tr. pallidum*. The results are here reported.

Methods and Material.

Two experiments are reported. The series of animals of the first experiment comprised seven rabbits with a complete thyroidectomy, six with a partial thyroidectomy, and six controls. They were inoculated on January 26, 1923. The opera-

tions were performed 7 and 10 days before inoculation in the case of complete removal of the gland, and 9 days in the case of partial removal. A second series were inoculated on October 29, 1923, and comprised five rabbits with a complete thyroidectomy performed 12 and 13 days previously, five rabbits with partial thyroidectomy performed 14 days previously, six rabbits with complete thymectomy performed 19, 21, and 24 days previously, and eight controls.

All operations were carried out under ether anesthesia. In the case of partial thyroidectomy, one lobe and the isthmus of the gland were removed; while the thymus was totally removed by a transthoracic operation (4, 5). At the end of the experiments, 4 months after inoculation of the first series and 7 months after that of the second, all animals were killed with ether and a complete autopsy performed. A search for thyroid or thymus fragments was made at this time. In two instances a tiny nodule of thyroid tissue was found.

The rabbits were young adult or adult males, matched according to age and breed from a selected stock. They were separately caged and fed a standard diet of hay, oats, and green vegetables.

The strain of *Tr. pallidum* used was the Nichols strain, isolated in 1912, and carried in this laboratory since 1917. Inoculations were made in one testicle with 0.2 cc. of a salt solution emulsion rich in treponemata as determined by dark-field examination. The tissue used for the emulsion was derived from an actively progressing orchitis.

The effect of the various operative procedures was studied in relation to the clinical course of the disease. Each rabbit was examined at frequent intervals and notes made of the general physical condition and disease manifestations. Particular attention was paid to the incubation period, the type and duration of the primary orchitis, the involvement of the uninoculated testicle and of the epididymides and scrota, and the occurrence and course of lesions in remote parts of the body such as the bones and periosteum, the skin and mucous membranes, and the eyes. The duration of the experiments, 4 and 7 months, was determined by the fact that, in the majority of instances after testicular inoculation, healing of syphilitic lesions other than minor residual lesions of the genitalia occurs within 4 months, although certain rabbits may continue to show persistent or recurrent lesions, particularly of the eyes and skin, for longer periods.

The results of the experiments are summarized in a series of tables of which Tables I and III contain abbreviated clinical records of individual animals. A general classification of the grades of infection is given in Table II, with the terms slight, fair, moderate, well marked, and severe. In Tables IV and V we have attempted to indicate graphically the character of the infection of each animal as determined by the lesions developed. Four sizes of solid circles are used. The smallest circle indicates a slight lesion or one of short duration, and in the case of extensions and secondary manifestations, few or transient lesions. The next larger circle indicates a more pronounced lesion or a slight one of long duration or more numerous, more marked, or more lasting extensions and secondaries. The more severe lesions, either of grade, number, or persistence, are designated by the

third and fourth size circles. For convenience, scrotal involvement has been grouped with lesions of the epididymides under the heading of extensions. In the last column of Tables IV and V, the grade of infection of each animal, as shown by the manifestations of the disease, has been designated by a series of one to five plus signs signifying a slight to severe condition.

RESULTS.

The results of the experiments are tabulated in detail so that only the essential features will be referred to.

Considering first the control animals, it is evident that the disease in the first series, Table I, was definitely more severe than in the second, Table III. Such differences in experimental syphilis of the rabbit are not infrequently encountered and, as will be seen later, can definitely influence the results of a particular experiment. Furthermore, well marked individual variations in the disease practically always occur in any single group of five to ten animals. The disease of the control rabbits of the first experiment may be described as of average grade taking into consideration both the genital[1] and the generalized lesions (Tables I and IV). Generalized manifestations occurred in five of the six rabbits (84 per cent) and numbered 25 definite lesions, 5 of the bones and periosteum, 18 of the skin, and 2 of the eyes.

The experiment was ended 4 months after inoculation, at which time active but non-progressive syphilitic lesions of the genitalia were still present in four rabbits and, in addition, the subsiding keratitis in two of these animals could still be seen (Table I). Two of the group had been free from any clinical manifestations for 9 and 14 days respectively. In this control group of six rabbits the infection taken as a whole may be termed moderate to well marked in three, fair in one, and slight in two animals respectively (Table II).

The disease which developed in the animals in which a complete or partial thyroidectomy (Groups A and B, Table I) had been

[1] We have not attempted to separate what might properly be considered true metastatic lesions of the scrota from those which develop as extensions from lesions of the testicle or tunics or from those occurring at the site of needle puncture at the time of testicular inoculation. Some involvement of the epididymis probably always occurs as a direct extension from the testicular process, and frequently the lesions persist after regression and healing of the orchitis. We have included only those instances which were clinically unmistakable.

TABLE I.

Course of Infection. Experiment 1.

Group and No. of rabbit.	Incubation period.				Incubation period and No. of lesions in remote sites of the body.										Freedom from lesions.	Postmortem examination.	
	Orchitis.		Scrotal lesions.		Bones and periosteum.		Skin.		Mucous membrane.		Eyes.		Total No.			Time after inoculation.	Residual syphilitic lesions.
	Primary.	Metastatic.	Right.	Left.													
	days	*days*	*days*	*days*	*days*	(*No.*)	*days*	(*No.*)	*days*	(*No.*)	*days*	(*No.*)			*days*	*days*	
A																	
1	10	39									98	(2)	2		0	117	Left orchitis, periorchitis, and epididymitis. Right and left keratitis.
2	14	35					73	(1)					1		15	123	Negative.
3	10	35		73	45–66	(8)	45–112	(12)	73–98	(2)	73	(2)	24		0	125	Left orchitis. Right and left keratitis. Lesions of bones and skin.
4	10	35	35		47–52	(6)	47–68	(28)	68	(1)	73	(2)	37		0	116	Right scrotal lesion.
5	10	32	34		45–61	(4)	55	(2)			81	(2)	8		10	122	Negative.
6	10	35	52	68					73	(1)			1		0	123	Right periorchitis. Right scrotal lesion. Left orchitis, periorchitis, and scrotal lesion.
7	10	44											0		24	118	Negative.
	10.5 (av.)	36.4 (av.)				18		43		4		8	73		16.3 (av.)		

B														
1	24	55	46								0	20	118	Negative.
2	21	52	35				61	(1)			1	20	118	"
3	21	44	61								0	0	123	Right epididymitis?
4	29	59									0	25	123	Negative.
5	21	59	39				55	(1)		103 (1)	2	8	119	"
6	21	61			61	(1)					1	35	116	"
	22.8 (av.)	55.0 (av.)				1		2		1	4	21.6 (av.)		
C														
1	10	48									0	9	117	Negative.
2	24	46	52	83						115 (1)	1	0	123	Right epididymitis. Right keratitis.
3	10	46	61		55–68	(2)					2	0	123	Right orchitis, periorchitis, and epididymitis. Right scrotal lesion.
4	14	44	55	89	61	(1)	55–89	(9)		119 (1)	11	0	122	Right orchitis, periorchitis, and epididymitis. Left orchitis. Left keratitis.
5	10	44	32	44			55–68	(4)			4	14	119	Negative.
6	7	41	52		53–68	(2)	55–80	(5)			7	0	116	Right and left epididymitis.
	12.5 (av.)	44.8 (av.)				5		18		2	25	11.5 (av.)		

Group A. Complete thyroidectomy.
" B. Partial "
" C. Controls.

TABLE II.

Classification of Grade of Infection.

	Complete thyroidectomy.	Partial thyroidectomy.	Complete thymectomy.	Controls.
Experiment 1.				
Severe, +++++	2 (Nos. 3, 4)			
Well marked, ++++				1 (No. 4)
Moderate, +++	1 (No. 5)			2 (Nos. 5, 6)
Fair, ++	3 (Nos. 1, 2, 6)	1 (No. 5)		1 (No. 3)
Slight, +	1 (No. 7)	5 (Nos. 1, 2, 3, 4, 6)		2 (Nos. 1, 2)
Experiment 2.				
Severe, +++++				
Well marked, ++++	1 (No. 1)			
Moderate, +++	2 (Nos. 4, 5)			1 (No. 8)
Fair, ++	2 (Nos. 2, 3)	1 (No. 1)	2 (Nos. 1, 4)	2 (Nos. 4, 7)
Slight, +	1 (No. 6)	4 (Nos. 2, 3, 4, 5)	4 (Nos. 2, 3, 5, 6)	5 (Nos. 1, 2, 3, 5, 6)

performed shortly before inoculation differed in many respects from that of the controls. Considering first the complete thyroidectomy group, the disease as a whole was much more marked. The average incubation period of the primary orchitis was 2 days shorter than in the controls, while the metastatic orchitis developed 8 days earlier and the general character of the genital lesions was generally more severe than in the controls.

A most striking difference in the completely thyroidectomized rabbits was in the generalized lesions (Group A, Tables I and IV).

There were 73 secondary lesions among six of the seven animals of the group; that is, twice as many per animal as in the controls. They were distributed as follows: bones and periosteum 18, skin 43, mucous membranes 4, eyes 8. Their time of appearance was generally earlier than in the controls, and, in addition, their duration was longer, as shown by the presence of a number of lesions 4 months after inoculation (Table I, last column).

The general character of lesions in the completely thyroidectomized rabbits was rather peculiar. The primary and metastatic orchitis and many cutaneous granulomata, including those of the scrota, were extensive processes with an unusual degree of boggy induration which gradually became extremely hard. Regression and resolution of all lesions proceeded slowly, and, in the case of the skin granulomata, there was a noticeable tendency toward the persistence of dry, indolent, skin nodules or patches of thickening. Renewed activity of all types of lesions with a return of progressive growth is not infrequently observed in normal rabbits but this characteristic was more noticeable in the thyroidectomized animals and contributed to the longer duration of the clinical manifestations of the disease.

At the end of the experiment, four thyroidectomized rabbits still showed active syphilitic lesions while the residual lesions in the control animals were less marked and were, for the most part, regressing. The relative proportion of the various grades of infection, as seen in Table II, was about the same as in the controls, but in two operated rabbits, Nos. 3 and 4, the disease was very severe. No other rabbit in the entire experiment approached these in the number, variety, and severity of generalized lesions.

The group of partially thyroidectomized rabbits (Table I, Group B) showed a disease picture quite different from that of either the controls or completely thyroidectomized animals in that the manifestations of the infection were of an extremely mild or benign type. The incubation period of both the primary and metastatic orchitis

TABLE III.

Course of Infection. Experiment 2.

Group and No. of rabbit.	Incubation period.				Incubation period and No. of lesions in remote sites of the body.								Freedom from lesions.	Postmortem examination.	
	Orchitis.		Scrotal lesions.		Bones and periosteum.		Skin.		Mucous membrane.		Eyes.		Total No.		
	Primary.	Metastatic.	Right.	Left.										Time after inoculation.	Residual syphilitic lesions.
	days	*days*	*days*	*days*	*days*	(*No.*)	*days*	(*No.*)	*days*	(*No.*)	*days*	(*No.*)		*days*	*days*	
A															
1	9	39			54–73	(8)			149	(1)	73	(2)	11	29	220	Negative.
2	14	35	44		59–63	(4)			154	(1)	73–86	(2)	7	24	220	"
3	14	38			65	(1)	154	(1)	141–154	(2)			4	0	220	Penis. Anus.
4	14	39	42		59	(1)			167	(1)	80–170	(2)	4	0	220	Right periorchitis and epididymitis. Left periorchitis. Anus. Necrosis of nasal bone.
5	14	38	32	127	54–59	(3)					88	(2)	5	0	220	Right and left periorchitis.
6	9	32	44										0	0	220	Left epididymitis.
	12.3 (av.)	36.8 (av.)				17		1		5		8	31	26.4 (av.)		
B															
1	21	54	28	77	73	(1)	89–127	(6)					7	30	221	Negative.
2	18	50	73										0	115	221	"
3	18												0	122	221	"

	4	21			59							0	97	221	Negative.
	5	18	51				54	(1)		66–77	(2)	3	115	221	"
		19.2 (av.)	51.6 (av.)			1		7			2	10	95.8 (av.)		
C															
	1	18	47			63–73	(4)					4	47	224	Negative.
	2	9	?									0	87	224	"
	3	18	47							89–127	(1)	1	70	224	"
	4	18	42			63	(1)	63–98	(3)			4	70	224	"
	5	18	59	73								0	119	224	"
	6	21	54									0	126	224	"
		17.0 (av.)	49.8 (av.)			5		3			1	9	86.5 (av.)		
D															
	1	18	42							149	(1)	1	23	219	Negative.
	2	14	42	59								0	114	219	"
	3	14	35									0	96	219	"
	4	14	38	32	73	59	(1)	59	(1)			2	96	219	"
	5	14	47					59	(1)			1	121	219	"
	6	32	50	44						175	(1)	1	37	219	"
	7	14	?			59–63	(2)			119	(1)	3	97	220	"
	8	14	42	77		59–73	(2)	98	(5)	102	(1)	8	83	220	"
		16.7 (av.)	42.2 (av.)			5		7			4	16	83.3 (av.)		

Group A. Complete thyroidectomy.
" B. Partial "
" C. Thymectomy.
" D. Controls.

was significantly longer (Table I, Group B), and the genital lesions, as a whole, were less marked and of shorter duration than those in the other two groups (Table IV).

The mild character of the infection in these partially thyroidectomized rabbits is particularly well illustrated by the extremely small number of generalized lesions—only four—which occurred in three of the six animals (Tables I and IV). In one rabbit there was a small skin granuloma, and in another, one bone lesion; while in the third animal there was a skin granuloma and a unilateral keratitis. These lesions developed at about the same time as those of the controls but were of relatively short duration and all were healed before the end of the experiment.

There was one rabbit in the partial thyroidectomy group, No. 5, with an infection which has been classified as of fair grade, comparable to that of No. 3 in the controls or of Nos. 1, 2, and 6 in the completely thyroidectomized group, while the disease of the other five rabbits was only of a slight grade (Table II).

In the second experiment the differences in the disease picture of the various groups are somewhat less striking, perhaps because the infection itself was on a lower plane of severity.

This is shown by the longer incubation period of the primary orchitis (Table III), the less pronounced character of this and other genital lesions, and the fewer number of generalized manifestations (Table V). There were 16 secondary lesions distributed as follows: bones and periosteum 5, skin 7, eyes 4. The bone lesions and one of the skin occurred in the usual time, that is 2 months after inoculation, but five skin granulomata developed much later than usual (Rabbit 8), as was also the case with three of the four instances of a keratitis.

This experiment lasted 7 months and at its termination no residual syphilitic lesions in the control rabbits were found clinically or in the gross at postmortem examination. The average time in which there had been no clinical manifestations of the disease was 83 days. In classifying the types of infection shown by these rabbits, one animal, as in the first experiment, No. 8, is graded as having a disease of moderate severity, in two, Nos. 4 and 7, it was fair, while in the remaining five it was of slight degree (Table II).

There were six rabbits from which the thyroid gland had been removed before inoculation. As in the case of Experiment 1, the disease

was again more severe in them than in the controls. The incubation period of the primary and metastatic orchitis was shorter than in the controls, and the genital lesions, as a whole, were more severe (Tables III and V).

TABLE IV.

Character of Infection as Determined by Lesions Developed.

Experiment 1.

Group and No. of rabbit	Genital lesions				Generalized lesions			Grade of infection
	Primary orchitis	Metastatic orchitis and peri-orchitis	Extensions: Scrota	Extensions: Epid-idymides	Skin and mucous membrane	Bones and periosteum	Eyes	
A 1	●	●		●			●	++
A 2	●	●		●	•			++
A 3	●	●	•	●	●	●	●	++++
A 4	●	•	●	•	●	●	●	++++
A 5	●	●	●	•	•	●	●	+++*
A 6	●	●	●	●	•			++
A 7	•	•		•				+**
B 1	•	•	●	•				+
B 2	•	•	●	•	•			+
B 3	•	•	•	●				+
B 4	•	•		•				+
B 5	●	•	●	●	•		•	++
B 6	•	•		•		•		+
C 1	●	•		•				+
C 2	•	●***	●	?			●	+
C 3	●	•	●	●		•		++
C 4	●	●	●	●	●	•	●	+++
C 5	●	●	●	•	•			+++
C 6	●	•	•	●	•	•		+++

Group A Complete thyroidectomy
" B Partial "
" C Controls

* Residual, hyperplastic thyroid nodule
** Extensive mange and wound abscess
*** Left testicle castrated 88th day

Generalized lesions developed in five of the six thyroidectomized rabbits (Table III).

There were 17 lesions of the bones and periosteum, 1 of the skin, 5 of the mucous membranes and mucocutaneous borders, and 8 of the eyes, a total of 31 as contrasted with 16 generalized lesions in six of eight controls. The lesions of the bones and periosteum developed at about the same time as those in the normal

animals, but the majority of eye lesions occurred much earlier; that is, within 73 to 88 days after inoculation. There were two lesions of the mucous membrane of the penis and three of the anus developing about 5 months after inoculation, and

TABLE V.

Character of Infection as Determined by Lesions Developed.

Experiment 2.

Group and No. of rabbit		Genital lesions				Generalized lesions			Grade of infection
		Primary orchitis	Metastatic orchitis	Extensions: Scrota	Extensions: Epididymides	Skin and mucous membrane	Bones and periosteum	Eyes	
A	1	●●●	●●●	•	●●	•	●●	●●	++++
	2	●●●	●●●	•	●●	•	•	●●	++*
	3	●●●	•	●●	●●●	•	•		++
	4	●●●	•	●●	●●●	•	•	●●	+++
	5	●●●	●●	●●	●●		•	●●	+++
	6	●●	•	•	●●				+**
B	1	•	•	●●	•	●	•		++
	2	•	•	•	•				+
	3	•			•				+
	4	●		•					+
	5	•	•	•	•	•		●	+
C	1	●	•		•		•		++
	2	●	?		•				+
	3	•	•		•			•	+
	4	●	•		•	•	•		++
	5	●	•	•	●●				+
	6	●	•		•				+
D	1	●●	●●		•			•	+
	2	●●	•	•	•				+
	3	●●	•		•				+
	4	•	•	●●	•	•	•		++
	5	●●	•	•	•				+***
	6	•	•	•	•			•	+
	7	●●	?		•		•	•	++
	8	●●	•	•	•	•	•	•	+++

Group A Complete thyroidectomy
" B Partial thyroidectomy
" C Thymectomy
" D Controls

*Residual, pinhead thyroid nodule
**Multiple cutaneous and pulmonary abscesses
***Multiple pulmonary abscesses

again, as in the thyroidectomized rabbits of the first experiment, these were the only lesions of this class in the entire series. The one instance of cutaneous involvement developed in association with a lesion of the adjacent mucous membrane of the anus.

At the end of the experiment four rabbits showed residual syphilitic lesions, while none were found in any of the other animals of the series (Table III). In Rabbit 3 the regressing lesions of the penis and the anus were still present; the left epididymis of No. 6 showed a slight granulomatous enlargement; in Rabbit 4 there was a residual right and left periorchitis, a right epididymitis, some thickening of the anal mucosa, and a residual necrosis of the nasal bone; in Rabbit 5 there was an inactive bilateral periorchitis.

We have classified the infection of the thyroidectomized rabbits in this experiment as follows: well marked, 1; moderate, 2; fair, 2; and slight, 1, as shown in Table II.

The disease of the five partially thyroidectomized rabbits was, as in the first experiment, of a more benign character than that of the controls.

The primary orchitis was not as severe and the average incubation period was 3 days longer (Tables III and V). Furthermore, the orchitis of all the partially thyroidectomized rabbits was not clinically apparent before 18 to 21 days, while it was definitely so in six of the eight controls within 14 days. The metastatic orchitis in seven of the eight controls developed after an average incubation period of 6 weeks, but among the five partially thyroidectomized rabbits a similar lesion had occurred in only three after 51 days.

Generalized lesions were found in only two of the five rabbits comprising this group, an animal incidence of 40 per cent as compared with an incidence of 75 per cent in the controls (Table III).

There were 10 such lesions, 1 of the bones and periosteum, 7 of the skin, and 2 of the eyes, and 7 of them occurred in one rabbit, No. 1, obviously the least resistant animal of the group. Rabbit 5 had one cutaneous granuloma and a bilateral keratitis which developed in the unusually short time of 2 months after inoculation.

The duration of both the genital and secondary lesions in this group was relatively short, as is shown by the length of time preceding the close of the experiment in which no lesions were found; that is, 30 to 122 days, or an average of 96 days. In the case of the controls this period was 83 days, while it was but 27 days for the only two completely thyroidectomized rabbits which were negative at the conclusion of the experiment.

In classifying the grades of infection of individual animals in the group, as shown in Table II, we have considered the disease of Rabbit 1 as representing a fair infection and that of the remaining four as slight.

The last division in the experiment consists of a group of six rabbits in which the thymus gland had been completely removed prior to inoculation (Table III, Group C). The infection which developed in these animals was, in general, like that of the partially thyroidectomized rabbits, less severe than in the controls, yet with certain differences.

The primary orchitis was of the same order as that of the controls, but the metastatic orchitis developed a week later and was slightly less pronounced (Tables III and V). By referring to Tables III and V it will be seen that the primary orchitis and the epididymitis of the thymectomized and control rabbits were, on the whole, quite similar, but that as regards the metastatic orchitis and scrotal involvement the thymectomized rabbits more nearly resembled the partially thyroidectomized animals.

As concerns generalized lesions, the thymectomized rabbits were also more like the partially thyroidectomized animals.

There was almost the same total number of lesions, that is nine in one and ten in the other; these occurred in two of five partially thyroidectomized and in three of six thymectomized rabbits. The lesions were distributed as follows: bones and periosteum 5, skin 3, eyes 1 (Tables III and V). Their time of appearance, general character, and duration presented no unusual features.

At the conclusion of the experiment all thymectomized animals had been clinically free from syphilitic manifestations for 47 to 126 days, or an average of 87 days. In this respect the group resembles the controls with an average figure of 83 days rather than the partially thyroidectomized animals whose average time was 96 days.

The degree of infection of the individual rabbits of the group has been classified as follows: fair, 2; slight, 4; as shown in Table II. This grouping resembles that of the partially thyroidectomized rabbits.

DISCUSSION.

The experiments reported indicate that, in rabbits, surgical removal of the whole or a part of the thyroid gland or of the entire thymus gland

shortly before inoculation with *Tr. pallidum* is followed by well defined differences in the clinical manifestations of the disease. In the case of complete thyroidectomy, the effect was, in general, one of increased severity as manifested especially by the shortened incubation period and pronounced grade of both the primary and metastatic orchitis, the much higher incidence of generalized lesions, and the distinct tendency for all lesions to be more enduring than in the control animals or, it may be added, than is ordinarily the case in normal rabbits. Partial thyroidectomy, on the other hand, resulted in a disease that was generally less severe than that of the controls as shown by the milder character of the primary and metastatic orchitis but especially by the low incidence of generalized manifestations and by the relatively short duration of all the lesions. These contrasting effects of complete and partial thyroidectomy occurred in both experiments, although, as has been pointed out, they were more marked in one than in the other.

It is not possible to speak so definitely about the effect induced by ablation of the thymus since it was studied in but one experiment and, as it happened, the less favorable one for a demonstration. The disease which developed in the group of rabbits with a complete thymectomy was of a mild type, much less severe than in the group of completely thyroidectomized animals and, on the whole, somewhat less so than that of the controls. This was particularly evident in the metastatic lesions of the genitalia and the number, incidence, and distribution of generalized manifestations. In many respects the general plane or grade of infection was similar to that of the group of partially thyroidectomized rabbits.

Because of the variations in the character of syphilitic lesions shown by individual animals in any series of five to ten rabbits, we have discussed the effects induced by various surgical procedures on a group basis. However, the disease manifestations in certain rabbits were of particular interest.

In the first set of completely thyroidectomized rabbits there were two animals (Table I, Group A, Rabbits 3 and 4) and in the second set there was one (Table II, Group A, Rabbit 1) in which the disease was considerably more severe than in any other animal of either series.

In the second experiment, there was one rabbit (Table III, Group B, Rabbit 1)

in which partial thyroidectomy was followed by more marked disease manifestations than in the other rabbits of this group. A somewhat similar instance is that of Rabbit 4 in the thymectomy group (Table III, Group C). In both animals there was but one bone lesion, a fact which would indicate that at this time the resistance of the host was on a comparatively high level. Subsequently, however, this level was lowered sufficiently to allow the development of skin granulomata, six in the case of the partially thyroidectomized and three in the thymectomized rabbit. Since no further lesions developed it may be presumed that the plane of resistance again was raised, probably in connection with the occurrence and healing of the skin lesions. The disease picture of these two rabbits should be compared with the similar one of Control Rabbit 8 (Table III, Group D). In this animal there were two lesions of the bone and five of the skin but the resistance of this animal, unlike that of the partially thyroidectomized and thymectomized rabbits mentioned above, was not sufficient to prevent the subsequent development of a keratitis. In other words, the least resistant control animal was less resistant than the least resistant partially thyroidectomized and thymectomized rabbits.

There are several phases of this work which are significant from the standpoint of the biology of syphilitic infections. It is evident that the general character or severity of the disease at the time of the experiment is a factor which may influence such effects as are induced by the various operative procedures employed. Thus, when the severity of the disease is of average grade, as in the first experiment, the effect of complete or partial removal of the thyroid is striking, as is shown graphically in Table IV. Syphilitic lesions were much more pronounced in the group of rabbits in which the thyroid had been completely removed than in the control group. The effect of a partial thyroidectomy, on the other hand, was in the opposite direction, the disease being less severe than in the controls. When, however, the disease was pursuing a relatively mild course with fewer generalized lesions, as in the second experiment, the effects of complete or partial thyroidectomy were less conspicuous (Table V).

If one considers experimental syphilis of the rabbit as largely determined, in its manifestations, by the reaction and resistance of the host, either natural or acquired, then it is evident that animal resistance was on a higher plane in the second experiment than in the first. In such rabbits as those of the second experiment, factors which would operate toward a depreciation of resistance might not be able to produce this effect to the degree that would be possible under inherent or so called "natural" conditions of lower resistance. On the other

hand, factors which tend to increase the forces of defense or to render them more efficacious might be reflected in a disease little if at all milder than that which would actually develop in such initially resistant animals.

In this discussion of the relationship between the manifestations of the disease and the resistance of the experimental animal, one other point may be briefly referred to. It has been shown that the occurrence of various manifestations of the disease as well as their severity and duration bear a definite relationship to one another (6). Lesions of the eyes occur most frequently in cases of severe syphilis or in animals in which previous lesions have been comparatively slight or of relatively short duration; they rarely occur in animals that show a prompt and vigorous reaction. It may be added that eye lesions are usually terminal events in the manifestations of the disease, and that the most common type is a keratitis. Extensive cutaneous involvement, save in cases of malignant syphilis, is usually associated with minor bone lesions or none, and this condition has been interpreted upon the basis that the resistance of the animals, which was sufficiently high following the primary orchitis to prevent the development of bone lesions, was not sufficient to afford protection to the skin. The occurrence of bone lesions, however, indicates that the resistance of the animal is not high at the time of their development. If no cutaneous lesions develop, a sufficient resistance may be assumed to have taken place in association with the development and course of the preceding lesions.

The primary reaction of the completely thyroidectomized rabbits was unusually prompt and vigorous, but it was followed by numerous secondary manifestations including a large proportion of eye lesions. There were eight instances of eye involvement among four animals of each experiment. Among the partially thyroidectomized rabbits, on the other hand, although the primary reaction was, in general, slight, the subsequent course of events was contrary to what might have been expected in normal animals. In the first experiment there was only one lesion of the bones and two of the skin, but instead of a fair number of eye lesions subsequently only one developed, indicating the persistence of a high state of resistance. In the second experiment there was again one bone lesion but seven of the skin, six of which

occurred in one animal,—the two instances of eye lesions developed in the animal that had previously a single granuloma of the skin. While the proportion of eye lesions in the partially thyroidectomized rabbits of the second experiment is practically the same as in the controls, they were distributed in the control group among four animals, an animal incidence of 50 per cent, whereas in the operated group they were limited to one, an animal incidence of 20 per cent.

In the thymectomized group, somewhat different conditions prevailed. The primary reaction had a closer resemblance to that of the controls than to that of the partially thyroidectomized rabbits, but the generalized manifestations were less marked especially as regards animal incidence. However, there was only a single instance of eye involvement in the thymectomized group.

The percentages for the proportion of eye lesions which developed in the several groups of both experiments, figured upon a basis of possible numbers, is as follows: complete thyroidectomy, 61.5 per cent; partial thyroidectomy, 13.6 per cent; thymectomy, 8.3 per cent; controls, 21.4 per cent. In other words, a high proportion of eye lesions in a group of animals with other syphilitic lesions of considerable severity indicates a state of low resistance, while few eye lesions in rabbits with other manifestations of a minor character indicates a high level of resistance.

It is significant that the operative procedures employed did not produce identical effects in every rabbit of a series; that is, individual variations in the clinical manifestations of the disease occurred as in normal animals. It would appear, therefore, that our interference with the thyroid or thymus glands merely altered the reaction of the host on whatever plane or in whatever state it happened to be. From one standpoint the most outspoken effect was obtained with complete removal of the thyroid. This tended in the direction of lowered resistance resulting in a more severe disease. In the case of a malignant neoplasm with which we have worked (2) this effect practically always occurred and was very striking. The less constant effects in syphilitic infections are probably to be explained by the difference in type of the two diseases. In normal rabbits the neoplastic disease is acute or subacute and a certain proportion of deaths occur from widespread metastatic involvement as early as 3 to 4 weeks after

inoculation; while experimental syphilis of the rabbit is essentially a chronic condition, in which secondary lesions in remote parts of the body do not usually occur for about 2 months and which, in the great majority of cases, tends toward complete recovery within 4 to 6 months. Thus, in the case of experimental syphilis there is an opportunity for physiological readjustments between the time of inoculation and the development of generalized lesions which is not present in the case of the tumor. If, on the other hand, such readjustments or compensations are not forthcoming in the case of a syphilitic infection, the more marked generalized manifestations of this disease are comparable to the higher incidence and widespread distribution of metastases seen in the malignant disease of thyroidectomized rabbits. That the factor of time enters in is brought out by the character of the primary and metastatic orchitis, which in both of the experiments here under consideration were more pronounced in the completely thyroidectomized groups than in the controls.

There is some indication that even individual animal variation may have been influenced to some extent by our procedures. The infection was in no instance more than of slight or fair grade in the instances of partial thyroidectomy and perhaps also in those of complete thymectomy, whereas in the controls of the first experiment half of the rabbits had a moderate or well marked infection and in the second experiment there was one such instance (Table II). While it is not impossible that all the rabbits in the partially thyroidectomized and thymectomized groups were naturally of high resistance, the chances are that at least one in each group was less resistant than the others and if not interfered with would have developed a moderate or well marked grade of infection.

It would appear from these experiments that, in the rabbit, surgical removal of the thyroid and of the thymus is followed by alterations in the clinical manifestations of experimental syphilis. We interpret such alterations as a consequence of changes in the mechanism of the host's reaction or resistance to the infection. From this point of view the experiments furnish additional evidence in support of the conception referred to in the beginning of this paper, that the integrity and balance of the system of glands of internal secretion play an important part in the reaction of the host to disease conditions.

SUMMARY AND CONCLUSIONS.

Experiments are described in which the thyroid or thymus gland of rabbits was removed prior to inoculation of the animals with *Tr. pallidum*. The effect of these procedures is described from the standpoint of the manifestations of the disease. After complete thyroidectomy, the disease was considerably more severe than in the controls and very markedly so in certain instances. Partial thyroidectomy, on the other hand, resulted in a milder disease than that of the controls. The effect of complete thymectomy was less pronounced than that of either complete or partial thyroidectomy, but, in general, the syphilis resembled that in partially thyroidectomized animals.

These effects are discussed in relation to the host's reaction and resistance to experimental syphilis and the conclusion was reached that the integrity and balance of the glands of internal secretion play an important rôle in the mechanism of defense against this infection.

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VARIATIONS IN CO_2 REQUIREMENTS AMONG BOVINE STRAINS OF BACILLUS ABORTUS.

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In a former publication,[1] the importance of CO_2 above a certain minimum concentration in stimulating the multiplication of freshly isolated strains of *Bacillus abortus* was pointed out and the literature cited. CO_2 was shown to be necessary not merely as an adjuster of the hydrogen ion concentration but possibly as a source of carbon. In estimating the significance of this phenomenon it is necessary to keep in mind certain other characters of *Bacillus abortus*. This organism is aerobic, for without oxygen it fails to multiply. A continuing supply even of small quantities is sufficient. Growth in sealed tubes is always less abundant than in unsealed tubes after the strain has become even slightly saprophytized. On the other hand, the freshly isolated strain, which will be called the parasitic strain, grows poorly in air and soon dies out unless large numbers of living bacilli are deposited on the agar surface. Even then the growth remains focal. This localized multiplication was regarded as indicating a community effort in the production of CO_2 to favor the multiplication of at least one or a few of the bacilli deposited on the new substrate. Frequently the focal growths under the condition described, *i.e.* in open tubes to which large masses of bacilli have been transferred, appear first on the lateral margins of the slope as if some influence of the glass container was favorable. When tubes of NaOH were hung below the plug and the tubes sealed, growth frequently appeared first, if at all, between the agar and the glass. It is probably some association with a minute source and a reservoir of CO_2 that is here involved and this may

[1] Smith, T., *J. Exp. Med.*, 1924, xl, 219.

account for the original observation of Bang and Stribolt of two optima of growth in their deep, semisolid medium. They, however, refer them to two optima of O-tension.

EXPERIMENTAL.

The existence of strains departing more or less in their CO_2 relations from the more common type had already been demonstrated[1] and the next step to be taken was to determine the relative frequency of such strains and their possible relation to cultures used as vaccines. The strains which fail to grow in open tubes even in the presence of abundant seed and which fail in sealed tubes in high dilutions are the most common and widely distributed and may be considered the normal or type bovine strain.

The registering of differences toward CO_2 did not appear feasible since all strains grow well and even luxuriantly in the presence of small amounts of CO_2. It was therefore determined to use different dilutions of seed in tubes of nutrient agar hermetically sealed to measure the sensitiveness of freshly isolated strains to CO_2 as it accumulates to a given threshold value in the sealed tube. This method was described in the first paper. It remained to choose some definite series of dilutions and to standardize the method as far as possible without making it cumbersome. After various trials the following scheme was chosen for the routine testing of all freshly isolated cultures in 1924 and 1925.

Ordinary veal-infusion agar in quantities of 6 cc. sloped in test-tubes of $\frac{5}{8}$ to $\frac{3}{4}$ inch diameter was used for the initial culture. The condensation water was about $\frac{1}{3}$ cc. in volume and 1 cm. deep. After the original culture from fetus or inoculated guinea pig had reached its maximum growth a mass of bacilli was transferred with loop or wire and rubbed over the entire surface. In such a subculture properly sealed a filmy continuous growth appears within 2 or 3 days. This film is as a rule of nearly equal vigor in all sealed cultures, the seal favoring the parasitic and restraining the saprophytic types. The growth at the end of 2 to 3 days is washed down with the condensation water. This becomes heavily turbid. A few drops of bouillon should be added if the condensation water is not of the requisite depth.

This suspension is used to prepare dilutions. The writer had adopted the arbitrary scheme given below as requiring but little glassware. Two pipettes supplied with rubber "teats" or bulbs were drawn out so as to deliver 25 drops

per cc. A platinum loop was made of 24 gauge wire and 5 mm. external diameter and was found to deliver about 1/250 cc. of the suspension. The dilutions were made in 3 cc. bouillon:

(*a*) 1 loop of condensation water suspension to agar (control).
(*b*) $\frac{2}{25}$ cc. (2 drops) " " " " 3 cc. bouillon ($\frac{2}{75}$ dilution).
(*c*) $\frac{2}{250}$ cc. (2 loops) condensation water suspension to 3 cc. bouillon ($\frac{1}{375}$ dilution).
(*d*) $\frac{1}{25}$ cc. (1 drop) of (*b*) to 3 cc. bouillon ($\frac{1}{2,800}$ approximate dilution).
(*e*) $\frac{2}{250}$ " (2 loops) " (*b*) " 3 " " ($\frac{1}{14,000}$ " ").
(*f*) $\frac{8}{250}$ " (3 ") " (*c*) " 3 " " ($\frac{1}{140,000}$ " ").
(*g*) $\frac{2}{250}$ " (2 ") " (*d*) " 3 " " ($\frac{1}{1,054,000}$ " ").

One standard loop of each suspension in bouillon is transferred to sloped agar.

Two series may be prepared, one of unsealed and one of sealed tubes. Whoever undertakes this work will soon discover how far to go with these two series. Thus with the normal type of *B. abortus*, the growth in (*a*) unsealed is restricted to a few centers on the agar slope, or may appear only in the condensation water; (*b*) unsealed fails to develop. In the series of sealed tubes, (*a*) always develops, (*d*) rarely grows, and some strains fail to show any growth in (*c*). On the other hand, strains which have been under cultivation 2 or 3 years may multiply promptly not only in all sealed tubes but in all open tubes of the series and even several steps beyond the scheme as far as given. In the open series the cotton plugs must not be dipped in paraffin as this may act as a partial seal and modify the result.[2] The method as given may be placed on a decimal system of dilutions by using pipettes graduated for the purpose. It is important, however, to bear in mind that extreme accuracy and uniformity are not attainable in a biological test of this kind without an expenditure of time scarcely justified by the results to be obtained.

The sealing requires some care to eliminate small gaps and avoid cracks later on. Irregularities in the dilution series may be due to them. It has been customary to place a circular disc of thin asbestos on the cotton plug as a base and drop the

[2] In earlier work[3] it is stated that some strains grew in the second transfer without *B. subtilis*. This is probably to be accounted for by the procedure used in the writer's laboratory of dipping all plugs of agar tubes into paraffin to prevent too rapid evaporation. Sometimes the dipping was overdone and practically sealed the tube.

[3] Smith, T., and Fabyan, M., *Centr. Bakt., 1. Abt., Orig.*, 1912, lxi, 549.

sealing wax, softened in the flame, on it. A thin iron spatula heated in the flame is useful in smoothing the surface, filling gaps, and removing any laterally projecting edges which later on may be knocked off when tubes are handled. This accident may break the seal. The plug of sealing wax is removed by piercing it with a heated iron wire curved and pointed at one end. The tube is then heated in the flame to loosen the seal and the plug lifted out with the wire inserted in the hole. The layer of wax need not be thicker than 1 mm. if properly manipulated.

It has been assumed that the sealing tends to bring the CO_2 concentration to a given level which permits multiplication into visible colonies or films. The present paper brings no direct proof of this assumption. An indirect proof was brought by the following simple experiment.

Three agar slants inoculated with dilution (*b*) of a very slowly growing strain were prepared. One was left unsealed and the second was sealed. The third was connected by means of a curved glass tube and rubber connections to a 24 hour growth of a saprophytized strain of *B. abortus*. Within 2 days there was active multiplication in the third tube and in 3 days a very vigorous film. The sealed culture was free from visible growth on the 15th day. The unsealed tube remained as was anticipated permanently free from growth. In brief, the saprophytized strain was producing enough CO_2 to assist the parasitic strain to a vigorous multiplication besides supplying its own needs.

Results of the Sealing of Graded Dilutions.—The following data, unless otherwise indicated, were obtained from the first, or less commonly the second transfer from the original culture from fetus or inoculated guinea pig. It had already been stated that passage through a guinea pig does not modify appreciably the CO_2 relation. A number of determinations of strains isolated both from the fetus directly and from guinea pigs 1 to 3 months later are the basis for this statement. The differences encountered in the strains are best brought out by two illustrations.

Fetus 1175.—Culture from Fetus (lung).
In the open series: (*a*) One colony in 13 days.
In the sealed series: (*a*) Growth in 2 days; (*b*) in 8 days.
(*c*) and (*d*) No growth in 19 days.

Culture from Inoculated Guinea Pig.
In the open series: (*a*) One colony in 4 days.
In the sealed series: (*a*) Growth in 4 days; (*b*) in 5 days.
(*c*) No growth in 11 days.

Fetus 1187.—Culture from Fetus (fourth stomach).
Unsealed series: (*a*) Complete film in 2 days; (*b*) in 2 days.
Sealed series: (*a*) Growth in 2 days; (*b*) in 3–5 days; (*c*) in 2 days; (*d*) in 2–3 days.

To illustrate further the two types of growth, the data of 1924–25 have been brought together in Table I.

It will not be difficult to select the more saprophytic strains from this table. They are 736, 916, 1119, 1128, 1176, 1186, 1187, 1207, 1211, and 1214. The parasitic types either fail to grow at all in the unsealed tube (*a*) seeded with abundant fresh growth, or else only one or several colonies or growth centers appear and attain normal dimensions. In some (*a*) tubes a very fine film may be detected with a hand lens but it does not acquire naked-eye dimensions. In the saprophytic types a distinct, even rich film may appear in 1 to 2 days on (*a*), also on (*b*). In the sealed tubes growth on (*a*) fails to distinguish the two types. In the tube receiving a loop of dilution (*b*) growth also appears in tubes of both types, but much later in the parasitic type. The distinctions become clearly marked in (*c*) and higher dilutions. As a working guide sealed tubes should be kept in the incubator about 15 days although growth appears only rarely after the 10th day. The continued vitality of the transferred bacteria when growth fails to appear after 10 or more days has been tested by unsealing and placing them in a jar containing 2 to 5 per cent CO_2. The originally unsealed tubes frequently failed to grow at this time, whereas those kept sealed the same length of time developed into rich films in the CO_2 jar, depending on the degree of dilution. The slight drying of the unsealed medium may have killed the bacilli on the agar surface within the period mentioned. In unsealed tubes containing a large seed and but few growth centers up to 10 days, numerous additional colonies may appear in the CO_2 atmosphere. In the sealed and unsealed tubes, multiplication, if it goes on at all, takes place at first in the condensation water. A slight cloud-like deposit usually precedes by a day or two a vigorous surface crop of colonies. In the unsealed tubes this cloud in the condensation water may appear and remain the only sign of multiplication. In tubes of bouillon a distinction between the parasitic and saprophytic type may be seen in the clouding of the dilutions used to inoculate the slanted agar. Dilutions (*b*) and (*c*)

are clouded from the start because of the large number of bacteria added. Dilutions (*d*) and (*e*) become clouded in the saprophytic but

TABLE I.

Growth in Dilutions on Agar.

(The figures stand for the day on which growth appeared.)

Case No.	Immediate source of strain.	Unsealed.		Sealed.				
		a	*b*	*a*	*b*	*c*	*d*	*e*
735	Guinea pig.	1	2	—	4	6	10+*	—
916	" "	—	2	—	—	2	2	—
1114	" "	6 (1 colony.)	0	0	9	0	0	—
1119	" "	2	2	—	3	4–6	8+	—
1128	" "	1	9+	—	2	2	7+	—
1150	" "	4 (1 colony.)	—	2	13+	0	0	—
1167	Fetus.	0	0	2	7	13+	—	—
1167	1st guinea pig.	0	—	3	0	0	—	—
1167	2nd " "	0	—	6	10–12	0	0	—
1168	Fetus.	5 (3 colonies.)	—	3	5–9	0	0	—
1175	"	13 (1 colony.)	—	2	8	0	0	—
1175	1st guinea pig.	4 (1 colony.)	—	4	5	0	—	—
1175	2nd " "	0	—	2	7+	8	0	—
1176	Fetus.	1	—	1	2	4–5	5–6	—
1176	Guinea pig.	2	5	—	4	4	5	5
1186	" "	2+	6+	—	3–4	4	3	4–6
1187	Fetus.	2	2	2	3–5	2	2–3	6
1195	Guinea pig.	0	0	2	9	0	0	0
1202	Fetus.	11+	0	2–3	11	0	0	0
1205	Guinea pig.	7–8	—	3+	10–12	17	14	—
1207	Fetus.	1+	—	1	3	7	7	7
1211	"	2–5	—	1–2	5	5–6	7–8	—
1212	"	0	—	3–8	13–14	0	0	—
1214	"	4–5	0	1–2	6–9	6–9	8–9	—

* + signifies only one or a few colonies on the day indicated, followed later by richer growth.

not in the parasitic group. When placed in a CO_2 atmosphere the latter become clouded in 3 to 4 days.

The rate with which saprophytic characters are assumed in successive cultures will probably vary with a variety of conditions, such as rapid transfers, the quality of seed, sealing, refrigeration after growth has ceased, and others. Aside from these, certain inherent characters of the individual strain play a part. Although the test described for determining the CO_2 requirements readily separates cultures into two classes, these are only rough classifications and within each class individual differences are observable. To determine at what rate the changes favoring growth in open tubes takes place, a strain growing quite feebly in sealed tubes was chosen. Successive transfers were made by using dilutions equivalent to (*b*) of the scale and the tubes sealed. The first tube developed a film of growth in 10 days. The 7th transfer developed in 6 days. The 10th transfer, after a total period of 65 days, developed in 3 days; all still in sealed tubes. A series of unsealed tubes with cotton wool plugs dipped in paraffin was carried on parallel with the above. The material transferred from tube to tube was not diluted in order to insure one or more centers of growth. After 4½ months, a tube inoculated from a loopful of condensation water into which the entire film had been washed, denominated (*a*) in the scheme, developed in 5 days. In the original culture (*a*) failed entirely to multiply in such tubes. At this date the unsealed series appeared as follows: (*a*) 5 days, (*b*) 5 days, (*c*) 9 days. In the sealed series, (*b*) was 5 days, (*c*) 5 days, (*d*) 8 days. This particular strain was thus fairly well saprophytized in 16 transfers lasting 4½ months. The beginning of changes in the cultured strains is frequently indicated by several successive crops of colonies. A few colonies may appear early. Within 1 or 2 days a second, much larger crop of minute colonies appears. Rarely a third crop can be distinguished. These crops probably represent degrees of adaptation to the new conditions. The virulence of the freshly isolated strains whether in the parasitic or the saprophytic group was the same for guinea pigs. In other words, it was not possible to foretell from the lesions produced in what group the strain would fall.

Sources of the Saprophytic Types.—The strains of both parasitic and saprophytic types in Table I were isolated from animals comprising one large herd. Earlier single strains from four other herds all belonged to the normal or parasitic type. The evolution of the

saprophytic from the parasitic type remains to be determined. The writer has kept in mind the possibility that the saprophytic strains may have originated in cultures used as vaccines and that during several passages through cows they may have regained to a certain degree the original parasitic character, since they do not multiply as freely in open tubes as do vaccinal strains. The problem needs to be studied in a herd in which vaccination has not been practiced and which has not received accessions from vaccinated herds.

In the herd in question only one animal has been examined which gave birth to a premature calf 9 days after arrival from a distant state. The culture isolated was of the normal or parasitic type. In one heifer, the first pregnancy ended in abortion associated with a saprophytic type. The second also ended in abortion but a strain approaching the parasitic type was recovered. In the test, two crops of colonies appeared, suggesting a modification of the earlier saprophytic type towards the parasitic level. A. Harms[4] studied 13 strains of *B. abortus* with reference to the time required to bring them to multiply without association with *B. subtilis*. She found considerable variation, probably due in most instances to recovery of injected saprophytized strains.

In a study of abortions in several groups of heifers in the same large herd, it was evident that some one type was present in each group and passed from animal to animal.[5] In one group of 34 treated with killed cultures of *B. abortus*, only the normal type was recovered in 9 first or second pregnancies. From a second group of 9 treated with a saprophytized living culture, the normal type was recovered from two clinically normal pregnancies and the saprophytic type from the udder of a third cow a year later. A group of 13 heifers, controls to the preceding group, yielded the normal type from 6 animals. A year later one of the other seven yielded the saprophytic type. In a second control group of 10 heifers, the saprophytic type was recovered from 3. In a third control group of 14 heifers, the saprophytic type was found in 4. Thus in these two last groups the parasitic type was not found.

[4] Harms, A., *J. Bact.*, 1924, ix, 272.

[5] Smith, T., and Little, R. B., Studies in vaccinal immunity towards disease of the bovine placenta due to *Bacillus abortus* (infectious abortion), Monograph of The Rockefeller Institute for Medical Research, No. 19, New York, 1923, 87.

CONCLUSIONS.

Strains of *Bacillus abortus* freshly isolated vary in their CO_2 requirements. The origin and sources of the strains growing with less dependence on CO_2 (or sealing) may be in vaccinal strains or possibly in continued existence in the udder. The importance of these possibilities makes it desirable that all strains isolated should be subjected to some such test as is outlined in these pages.

FURTHER DATA ON THE EFFECT OF VACCINATION AGAINST BOVINE INFECTIOUS ABORTION.

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In a recent monograph[1] two experiments were described dealing with the effects of vaccination on the incidence of abortion in heifers associated with *Bacillus abortus*. The first experiment dealt exclusively with the effect of living cultures on the first and second pregnancies. In the second experiment one group of heifers was treated with cultures killed by heat. In the publication referred to only the first pregnancies were reported. There are now available data on the second pregnancies which shed some additional light on the value of vaccination.

The methods pursued were the same as those described in the monograph. All fetuses obtainable were cultured and at the same time guinea pigs inoculated with washings from uterine swabs. The agglutinins towards *Bacillus abortus* in the blood were measured by a suitably graded series of tests.

All animals were treated with vaccine before the first pregnancy only. In Group A all heifers received 4 doses of a heated culture, 2 before and 2 after the first service. Similarly the group treated with a living culture before the first pregnancy was not injected thereafter.

The outcome of first and second pregnancies is shown in Table I. It will be noted that between the two pregnancies some animals were disposed of as of reduced economic value.

Although Group A treated with dead cultures appeared to possess

[1] Smith, T., and Little, R. B., Studies in vaccinal immunity towards disease of the bovine placenta due to *Bacillus abortus* (infectious abortion), Monograph of The Rockefeller Institute for Medical Research, No. 19, New York, 1923.

considerable resistance when compared with the controls in the outcome of the first pregnancy, it failed to hold its advantage in the

TABLE I.

No. of pregnancy.	No. of animals remaining available.	Full-term pregnancies.		Abortions.	Per cent aborted.	Per cent aborted and with placenta diseased.
		Normal.	With placenta diseased.			
Vaccinated Groups.						
Group A. (Heated vaccine.)						
First.....	34	25	4	5	14.7	26.4
Second.....	30	23	2	5	16.2	23.3
Group B. (Living vaccine.)						
First.....	9	9	(3)*	0	0	(33.3)*
Second.....	9	9	0	0	0	0
Control Groups.						
Group Bc.						
First.....	13	6	0	7	53.8	53.8
Second.....	11	8	2	1	9.9	27.3
Group C.						
First.....	9	4	1	4	44.4	55.5
Second.....	4	1	2	1	25.0	75.0
Group D.						
First.....	14	9	1	4	28.5	35.7
Second.....	9	6	2	(1)†	(11.1)†	22.2
Summary of Controls.						
First.....	36	19	2	15	41.6	47.2
Second.....	24	15	6	2	8.3	33.3

* Normal births with *B. abortus* in uterine washings.

† Vibrionic.

second. Thus the actual abortion rate compared with the controls was as 14.7 to 41.6 per cent in the first and as 16.2 to 8.3 per cent in

the second. When we include full-term pregnancies with diseased placenta, the difference is, however, still in favor of vaccination, 23.3 to 33.3 per cent.

Group B treated originally with one dose of living cultures is of interest in having maintained complete resistance in the second pregnancy. In the first all pregnancies terminated normally, but in 3 of the 9 animals the uterine swab produced the characteristic disease in guinea pigs, although the placentas were normal. In the second parturitions, also normal, all uterine swabs failed to infect guinea pigs. Although in one animal the placenta was retained, guinea pig tests of uterine swab and scrapings of chorion were negative for *B. abortus*. Sections of the placenta of this animal failed to show lesions and the cause of the retention remained undetermined. The

TABLE II.

Time.	Normal pregnancies.	Abortions.	Adherent placentas.	Per cent aborted.	Per cent aborted and with adherent placentas.
1924					
Jan.....	39	5	2	12.8	18.0
Feb.....	42	8	5	19.0	30.9
Mar.....	37	6	6	16.2	32.4
Total.....	118	19	13	16.1	27.1

animals of this group had been distributed among older cows to increase the opportunities for infection. These results can scarcely be regarded as of no significance, even in view of the small number of animals in the group.

The heifers in the control groups, of which only two-thirds remained in the herd, show a marked reduction in actual abortions but a relative increase in full-term pregnancies with diseased, adherent placenta.

During 3 winter months when most of the parturitions in the vaccinated and control groups occurred, the abortion rate among the cows making up the remainder of the herd is given in Table II. There were no special tests made to determine the nature of the disease in these animals. The table represents older and seasoned animals. The total figures approximate closely those of the group which received the heated cultures.

CONCLUSIONS.

The partial protection afforded by four injections of a heated culture of *Bacillus abortus* of normal virulence during the first pregnancy is in part lost in the second. The superiority of a single injection of a living culture of relatively low virulence is evident in both pregnancies. In the experiment described, the protection was complete.

A RAPID METHOD FOR THE ISOLATION OF BACILLUS ABORTUS FROM UTERINE EXUDATE AND DISEASED PLACENTA.

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The diagnosis of contagious abortion in cattle not infrequently rests on the isolation of *Bacillus abortus* from infected material. The organisms may be present in such small numbers that repeated microscopic examination of films fails to reveal them. Uterine swabs and tissue from fetal membranes are usually contaminated with miscellaneous bacteria either from the utero-chorionic space or fecal matter or from both sources. The isolation of *Bacillus abortus* from such material is a matter attended with considerable difficulty. The preponderance of rapidly growing saprophytes together with the peculiar growth requirements of the microorganism itself make the use of simple plating methods for its recovery often very laborious and liable to failure and loss of material.

Animal inoculation has been resorted to as the most satisfactory method. Smith and Fabyan[1] showed, in 1912, that the injection of cultures of *Bacillus abortus* into the guinea pig gave rise to a disease of rare fatality, characterized chiefly by enlargement and congestion of the spleen together with foci of cellular proliferation. The splenic lesions began about the 3rd week, extended over a period of 10 to 20 weeks, and were followed by repair.[2] The disease could be transmitted from animal to animal by the injection of suspensions from a spleen in the acute stage. In 1918, Smillie[3] found that cultures could be obtained from the spleen 3 to 4 weeks after injection of the guinea

[1] Smith, T., and Fabyan, M., *Centr. Bakt., 1. Abt., Orig.*, 1912, lxi, 549.
[2] Fabyan, M., *J. Med. Research*, 1912, xxvi, 441.
[3] Smillie, E. W., *J. Exp. Med.*, 1918, xxviii, 585.

pig before definite gross lesions had appeared. The number of organisms in the spleen were found to be at their maximum between the 3rd and 4th week. After the 4th week a sharp decline in numbers was noticed.

These methods of isolation are reliable but time consuming, inasmuch as an interval of at least 3 weeks is necessary between injection of the guinea pig and cultivation from the spleen. An attempt to diminish the time interval before cultivation could be resorted to has led to the method to be described. The procedure is based on the observation made by Dr. Theobald Smith that the peritoneum of the guinea pig rapidly destroys most bacteria, excepting the spore stage, when injected in small numbers and it has been used in attempts to isolate certain bacteria in pathological material by timing the exposure in the peritoneal cavity. *Bacillus abortus* is unaffected by the normal defensive factors operative in the peritoneal cavity which are accountable in part for the sterilizing action.

Procedure.

The method employed is as follows:

A suspension in bouillon is made from the suspected material. If it is a uterine swab, it is rotated vigorously until a noticeable cloudiness results. If placental tissue is available, it is thoroughly washed in running water, scrapings from the diseased chorion or cotyledons removed to a small crucible or convenient vessel and ground with sterile sand. Bouillon is added and after subsidence of coarse particles the supernatant liquid is employed. Approximately 1.5 to 2 cc. of the suspension is injected intraperitoneally into each of two or more guinea pigs. If a preliminary microscopic examination of the material shows streptococci in abundance, it is well to reduce the dose to 1 cc. Guinea pigs weighing around 350 gm. are to be preferred.

After 5 days one animal is chloroformed, the abdomen exposed and the muscles thoroughly seared with a hot spatula. With strict aseptic precautions a medial incision is made and several cc. of bouillon introduced into the peritoneal cavity with a blunt and smooth tipped pipette. The cavity is washed out by withdrawing and expelling the bouillon a number of times. Roughly 0.5 cc. of the washings is added to each of several agar slants. The spleen is exposed, a bit of tissue torn off with sterile forceps and transferred to an agar slant. The cultures are placed in a closed jar in an atmosphere of 5 per cent carbon dioxide and incubated at 37°C. If *Bacillus abortus* is present growth either as isolated colonies or as a confluent film should occur in from 2 to 7 days. If reasonably strict aseptic

precautions are observed no contaminated tubes should be encountered. In case the cultures from the first animal remain sterile or are overgrown by bacteria other than *Bacillus abortus*, the second guinea pig should be killed and the procedure repeated. A third animal should be reserved and killed after 3 or 4 weeks.

Application of the Method.

The method described was tested with material from 12 cases of suspected bovine abortion. The results are presented below in tabular form. In addition, two cases from which *Bacillus abortus* was recovered are described in detail. Three and sometimes four guinea pigs were employed in order to ascertain roughly the length of time *Bacillus abortus* remained in the peritoneal cavity. The amount of growth on cultures from successive guinea pigs was simply estimated. Frequently isolated colonies were absent, growth occurring as a confluent film. Wherever possible the actual number of colonies is given.

Case 1176.—Uterine swab and a full-term fetus were received for examination. Autopsy of the fetus showed the presence of solid particles of meconium in the first and fourth stomachs. A pure culture of *B. abortus* was obtained from the lung, liver, first and fourth stomachs, spleen, and rectum. A culture from the kidney was without growth. Microscopic examination of films from the uterine swab showed microorganisms resembling *B. abortus*. 12 days later, the swab meanwhile being kept in the refrigerator, four guinea pigs were injected with 1.5 cc. portions of the swab suspension, three by the intraperitoneal route and one by the intrapleural route. Two guinea pigs were chloroformed after 5 days and cultures made from the washings of the peritoneal cavity in one case, of the pleural cavity in the other case. Spleen cultures were made from both animals. The turbid washings after subsidence showed many cells of which a small lymphocyte was the predominating type with smaller numbers of mononuclear and polynuclear leucocytes. There was evidence of phagocytosis in the two latter cell types. Cultures from the pleural washings showed in one case contamination, in others 35 and 6 colonies of *B. abortus*. The spleen culture showed a scant growth of *B. abortus*. Cultures from the peritoneal washings in one case showed contamination, in the others 2 and 4 colonies, respectively, while the spleen showed a scant growth. The third guinea pig was chloroformed 8 days after injection and cultures made from the peritoneal washings and from the spleen. The fluid was turbid and showed many lymphocytes with an occasional mononuclear and polynuclear leucocyte. 4 days later cultures from the washings showed 18, 10, and 12 colonies, respectively. The spleen gave a heavy confluent film. The fourth guinea pig was killed and cultured 14 days after injection. The washings showed a visible turbidity with cells in the same proportion as before. Cultures from the peritoneal

TABLE

Cultures from the Peritoneal Washing

Case No.	Nature of material.	Microscopic examination of films.*	Days after injection.	Abdominal washings.†				
1175	Uterine swab.		5	6	—	50		
			11	1	—	2	—	10
			17	0	—	0	—	2
			74					
			107					
1176	" "	+	5	4	—	2	—Contaminated.	
			8	18	—	10	—	12
			14	1	—	2	—	0
1185	" "	—	5	Contaminated.	—	0	—	0
			11	0	—	0	—	0
			21	Contaminated.	—	0	—	0
			28	0	—	0	—	0
1186	" "	—	6	Scant.	—	Scant.	—	Scant.
			10	"	—	"	—	"
			21	0	—	0	—	12
1187	" "	—	6	8	—	5	—	7
			11	2	—	6	—	4
			21	0	—	0	—	0
1191	" "	—	5	0	—	0	—	0
			12	0	—	0	—	0
			19	0	—	0	—	0
			26	0	—	0	—	0
1195	" "	—	7	0	—	0	—	0
			14	1	—	0	—	0
			21	0	—	0	—	1
1202	" "	+	3	Moderate.	—	Moderate.	—	Moderate.
			12	Scant.	—	"	—	"
			19	2	—	3	—	10

* + = *B. abortus;* − = *B. abortus* not found.

† The numbers represent colonies of *B. abortus.*

I.

Spleen of Injected Guinea Pigs.

Spleen.†	Order of guinea pig examination.	Remarks.
30	1st	*B. abortus* isolated from fetal organs.
100	2nd	
80	3rd	
7	4th	
65	5th	
Scant, confluent.	1st	
Heavy, "	2nd	" " " " " "
175	3rd	
0	1st	Abortion with retained placenta, fetus lost, agglutinins for *B. abortus* negative—probably vibrionic.
0	2nd	
0	3rd	
0	4th	
Scant, confluent.	1st	
Heavy, "	2nd	
Moderate, "	3rd	
18	1st	Cultures from fetal organs positive for *B. abortus*.
40	2nd	
150	3rd	
0	1st	*Vibrio fetus* isolated from fetal organs.
0	2nd	
0	3rd	
0	4th	
0	1st	Cultures from fetal organs negative.
24	2nd	
100	3rd	
Moderate, confluent.	1st	*B. abortus* isolated from fetal organs.
Heavy, "	2nd	
" "	3rd	

TABLE I.

Case No.	Nature of material.	Microscopic examination of films.*	Days after injection.	Abdominal washings.†				
1205	Uterine swab.		5	Scant.	—	Scant.	—	Scant.
			12	Contaminated.	—	Contaminated.	—	"
			19	0	—	0	—	"
1207	" "	—	5	Scant.	—	Scant.	—	10
			11	"	—	"	—	0
			18	0	—	0	—	Scant.
1211	Fetal membrane.	+	5	65	—	55	—	40
			13	Scant.	—	Scant.	—	0
			20	0	—	0	—	0
1214	Uterine swab.	+	5	Scant.	—	Scant.	—	Scant.
			12	"	—	"	—	"
			19	0	—	0	—	0

washings in one case gave no growth, in others showed 1 and 2 colonies, respectively. The spleen gave a pure growth of 175 colonies.

Case 1187.—Uterine swab and fetus were received for examination. Autopsy of the fetus showed the presence of solid particles of meconium in the fourth stomach. Pure cultures of *B. abortus* were obtained from the lung, first and fourth stomachs, and the rectum. Cultures from the spleen and liver were without growth. Microscopic examination of films from the uterine swab failed to show *B. abortus*. Three guinea pigs were each injected intraperitoneally with 1.5 cc. of a bouillon suspension made from the uterine swab. One guinea pig was chloroformed after 6 days and cultures made from the peritoneal washings and from the spleen. Cultures from the washings showed 8, 5, and 7 colonies, respectively, of *B. abortus;* the spleen showed 18 colonies. 11 days after injection the second guinea pig was killed and cultured. Cultures from the washings showed 2, 6, and 4 colonies, respectively, and from the spleen 40 colonies of *B. abortus*. 21 days after injection the third animal was killed and cultures made from the peritoneal washings, the liver, and the spleen. Cultures from the washings and the liver were without growth. The spleen culture showed 150 colonies of *B. abortus*.

DISCUSSION.

The injection of a mixed suspension of tissue fragments containing *Bacillus abortus* and other bacteria into the peritoneal cavity of the guinea pig is followed by a vigorous, differential sterilization whereby

—*Concluded.*

Spleen.†	Order of guinea pig examination.	Remarks.
Scant, confluent.	1st	Cultures from 2nd guinea pig, with one exception, showed *B. paratyphosus*.
Contaminated.	2nd	
Heavy, confluent.	3rd	
Moderate, "	1st	*B. abortus* isolated from fetal organs.
" "	2nd	
" "	3rd	
150	1st	
Moderate, confluent.	2nd	" " " " " "
" "	3rd	
Scant, "	1st	
65	2nd	" " " " " "
Heavy, confluent.	3rd	

after a few days and for a space of several weeks that microorganism is left in pure culture. After the 1st week, following injection, there is noted a gradual decrease in numbers. Reduction continues until sterility is reached, generally about the end of the 3rd week. During the same period of time there is a gradual increase in the number of microorganisms in the spleen. In no case, however, in the series studied were gross lesions in the spleen observable at autopsy. *Bacillus abortus* could be obtained in pure culture from the spleen as early as the 3rd day following injection and regularly on the 5th day. The cultures obtained from the spleen during this early period are probably due in part to the growth of organisms adhering to its surface. It is evident, however, that *Bacillus abortus* is carried very early into the circulation, and that growth is largely due to organisms actually in the splenic tissue. In one case, as noted in the case reports, the suspension was injected into the pleural cavity of a guinea pig and a pure culture of *Bacillus abortus* obtained from the spleen on the 5th day.

Cultures made directly from the blood gave irregular results. In one instance cultures made from the heart's blood 48 hours after injection were without growth. In another instance cultures made from the

heart's blood every 12 hours for 3 days after injection were all positive but with a scant growth. Cultures made from other organs, such as the liver, gave inconstant results. In one case the liver culture showed 2 colonies of *Bacillus abortus* while the spleen showed over 100. In two other cases the liver cultures were without growth while those from the spleen showed 150 and 25 colonies, respectively.

The factors accountable for the differential sterilization in the peritoneal cavity of the guinea pig were not studied. Undoubtedly many of the miscellaneous bacteria encountered are saprophytes and as such unable to adapt themselves to a parasitic mode of existence. Other bacteria, facultative parasites of a low order of virulence, must succumb to the normal defensive mechanism of the host. The injection is invariably attended by a cellular mobilization composed chiefly of small lymphocytes together with smaller numbers of mononuclear and polynuclear leucocytes. There is active phagocytosis, as evidenced by intracellular bacteria seen in stained preparations made from the peritoneal washings. It is to be anticipated that now and then streptococci or other pathogenic bacteria of sufficient virulence to overcome the normal defenses of the guinea pig may be present in the material employed for injection. Under these conditions a safe procedure would be to inject one or two guinea pigs subcutaneously. These usually survive even in the presence of streptococci or other pathogenic bacteria and may be cultured after the 3rd week.

SUMMARY.

A rapid method is described for the isolation of *Bacillus abortus* from the peritoneal cavity of the guinea pig following the injection of uterine exudate or placental tissue.

STUDIES ON THE RELATION BETWEEN TUMOR SUSCEPTIBILITY AND HEREDITY.

III. Spontaneous Tumors of the Lung in Mice.

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In discussing cancer from the standpoint of heredity the present tendency is to regard susceptibility to the development of tumors of each tissue or of each organ as if inherited separately. In a previous communication[1] from this laboratory evidence was presented in favor of the view that susceptibility to tumors of the mammary gland in mice is influenced by the laws of heredity and that it apparently behaves as a dominant though variable character. The present paper is concerned with the inheritance of susceptibility to tumors of the lung in mice.

Lung tumors as well as mammary gland tumors are not uncommon in mice. They have been reported by Livingood,[2] Tyzzer,[3] Jobling,[4] Haaland,[5] Slye, Holmes, and Wells,[6] and others. Tyzzer[3] states that growths appear to be more frequent in the lung than in any other organ. In 70 tumor mice which he examined, 83 neoplasms were found. 74 per cent of all the individuals had pulmonary tumors and 62 per cent of all the tumors were in the lung. A stock of mice which was under observation for 2 years yielded 12 mice (9 females and 3 males) with lung tumor from 500 autopsies. Haaland[5] found pulmonary tumors next in frequency to mammary tumors. Complete statistics are not given. In the

[1] Lynch, C. J., *J. Exp. Med.*, 1924, xxxix, 481.

[2] Livingood, L. E., *Bull. Johns Hopkins Hosp.*, 1896, vii, 177.

[3] Tyzzer, E. E., *J. Med. Research*, 1907, xvii, 155, 199; 1909, xxi, 479.

[4] Jobling, J. W., Spontaneous tumors of the mouse, Monograph of The Rockefeller Institute for Medical Research, No. 1, New York, 1910, 81.

[5] Haaland, M., *Ann. Inst. Pasteur*, 1905, xix, 165; *4th Scient. Rep. Imperial Cancer Research Fund*, London, 1911, 1.

[6] Slye, M., Holmes, H. F., and Wells, H. G., *J. Med. Research*, 1914, xxx, 417.

Slye stock[6] also lung tumors were found to be second to mammary gland tumors in frequency. 160 mice with pulmonary nodules were observed in 6000 autopsies (2.7 per cent) and one-third of all tumors found arose in the lung. A large number of cases have been discovered in this laboratory. As a result of the practice of making autopsies on all mice data have accumulated regarding the occurrence of pulmonary tumors in mixed populations and also in certain strains of mice that have been closely inbred. Since in many of the groups under observation a large number of the individuals of the more recent generations are not yet dead this preliminary report covers results from only a few of the earlier crosses in which the data are practically complete.

Several investigators have already published contributions to the subject of the hereditary aspect of tumors of the lung. Tyzzer[3] observed the tumor incidence in several inbred families of mice. In the largest group (Family A) there were 98 descendants belonging to several generations derived from an original mating of a mouse which never developed tumor with a female which was found to have a cystadenoma of the lung. Of 62 which died after reaching full maturity 20 developed one or more tumors, 17 being pulmonary tumors. If the mice of this family are classified according to whether they are the offspring of parents without tumors or of parents one of which developed a tumor, it is found that in the first group, 9 mice in a total of 67 (13 per cent) were tumor mice while in the second group 11 out of 28 (39 per cent) developed tumors. If only those individuals which lived more than 6 months are included in the estimates, among the 40 offspring born of tumor-free parents 23 per cent developed tumors while among 24 mice with one tumor parent 50 per cent developed tumors. Tyzzer concluded that although the numbers dealt with were small, the data tended to indicate that there is a stronger tendency to the development of tumors in the offspring of parents with tumors than in the offspring of parents which are free from tumors.

Slye, Holmes, and Wells[6] have published data from 6000 autopsies. In their stocks lung tumors do not ordinarily appear in mice younger than 12 months and about one-third of the individuals died under that age. 155 mice with lung tumors were investigated from the standpoint of heredity. Of these 146 were found to have a tumor ancestry but 9 of the cases of lung tumor appeared in mice without tumor ancestry and these 9 tumors were all benign. Since one-third of the mice autopsied were from non-cancerous strains, if inheritance played no part, there should have been 50 cases instead of 9 in these strains. The authors cited believe that in mice without cancer ancestry inflammatory reactions in the lungs seldom become neoplastic in character; that if excessive proliferation occurs in such individuals it does not exceed that of a slight papillary growth; and that heredity modifies the nature or degree of reaction to injury.

In the present paper no attempt has been made to classify the tumors according to the degree of malignancy shown by them. The histological aspect of tumors of the lung in mice was first described by

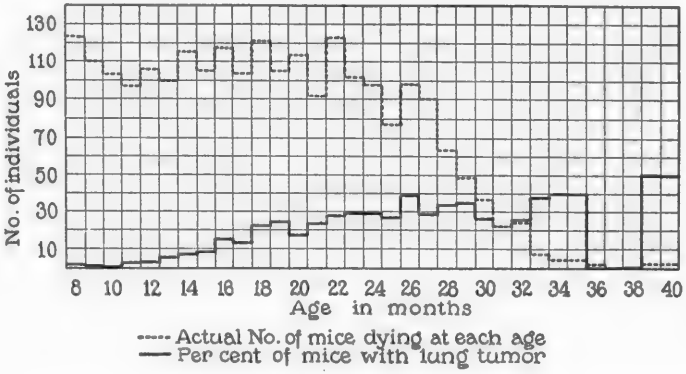
Livingood[2] and it has been the subject of so many communications since then that a detailed account is unnecessary here. A considerable variation exists in their behavior. Many show no evidences of malignancy while others appear to be actively growing, infiltrating the surrounding tissues in the lung or the mediastinum. They are often multiple. The neoplastic epithelium frequently resembles the bronchial epithelium, although examples in which it is like thickened alveolar epithelium are also found. We have encountered no tumors in this material like the epidermoid carcinoma of the lung described by Tyzzer. It is sometimes difficult to distinguish between neoplastic and inflammatory hyperplasia and several cases in our material have been classed as doubtful. Under this heading are also included a few mice in which pulmonary nodules were found at autopsy but in which postmortem changes made a histological verification impossible. The growths vary greatly in size. Sometimes they are so small as to escape detection at autopsy and are found only by chance in sections. Serial sections of the lung were not made so that in general only those nodules large enough to be visible to the naked eye are included in the data. The mice were allowed to live under conditions as nearly natural as a laboratory permits and the tumors under discussion were "spontaneous." Besides the lung tumors there were found in the material 7 sarcomas, 3 of which were in animals which also had lung tumors, and 8 epitheliomas of the jaw, 3 of which coexisted with tumors of the lung. One mouse with a lung tumor had a carcinoma in the ovary and 2 mice had carcinomas of the stomach. There were also a large number of tumors of the mammary gland. In fact the stocks of mice studied served as a source of supply for mammary neoplasms. The majority of the mice developing them were used in other experiments or disposed of outside this laboratory and so few of the records dealing with such animals, valid for the condition of the lung, are available that it was decided to omit them in the interest of a more uniform basis for conclusions. There are very few data from other laboratories regarding the percentage of lung tumors which occurred in the total number of mice with mammary gland tumors and also in the total number of mice free from such growths. Jobling, in a small series of mice with mammary neoplasms, found that pulmonary growths occurred in about one-third of the individuals while none appeared in several

thousand mice free from tumors of the mammary gland. The ages of these mice are not given. The few facts we have accumulated which bear on this point would tend to indicate that in our stock lung tumors coexist with mammary gland tumors in about the same per cent with which they occur in individuals free from mammary gland tumors. In an inbred strain in which the rate of incidence of lung tumors was 6.7 per cent among females free from mammary gland tumors there were 23 animals developing tumors of the mammary gland, in which the lungs also were examined. Only one of these had developed a primary pulmonary tumor.

The data which have been accumulated furnish information upon the rates of incidence of spontaneous tumors of the lung in mice (1) in relation to age and sex, (2) in two inbred strains of mice, (3) in the offspring from parents with and without pulmonary tumors, and (4) in the offspring from crosses between strains that have a high and a low rate, respectively, in regard to tumors in the lung.

1. Rates of Incidence of Spontaneous Tumors of the Lung in Relation to Age and Sex.—It has been noted that tumor rates vary markedly with the age of the individuals concerned although it is not clear whether this is a direct effect of the "age" of the tissues or indirectly due to the fact that a long life offers more opportunities for exposure to the various forms of chronic irritation which induce neoplastic disease. The earliest age at which a lung tumor has been found in these strains is 8 months and the greatest age is 40 months. They occur most frequently in mice of about 24 months or older. In one group of 1500 autopsies upon mice from the general stock the maximum rate of lung tumor incidence was 45 per cent at 26 months of age. In another group of about equal size the maximum rate of 38 per cent was reached at 29 months. The two groups taken together give a lung tumor incidence of about 17 per cent in 2300 mice which were over 7 months of age and 11 per cent in 3500 mice of all ages. These figures do not include mice with mammary tumors. We can follow the monthly fluctuation of the tumor rates in these two groups combined, in Text-fig. 1. This shows the actual numbers of mice dying at each age and the percentage of tumor mice occurring among them. At the younger ages (8 to 12 months) the tumor rate lies at the low level of 1 to 3 per cent. Beginning with the 13th month there is a gradual

increase in the rate and from the 22nd through the 29th month it is maintained at a level of about 30 per cent. At 26 months a rate of 39 per cent is shown and at 29 months it is almost as high. After the 29th month the rate declines slightly. The large percentages shown at 33 months and older are based on less than 10 mice in each group. If these individuals are classed together the incidence for the total number is 38 per cent, about the same rate as that given at 26 months. It is interesting to note that 2 mice died at 39 months and 2 at 40 months, and in each case 1 of the individuals was free from growths in the lung.



TEXT-FIG. 1.

Sex is probably not of importance in determining the occurrence of primary tumors of the lung. Slye, Holmes, and Wells report that 57.4 per cent of the lung tumors in their stock were found in females and 42.6 per cent in males. The reverse condition was found to exist in our stocks, as the higher percentage was shown by the males. Reference to Text-fig. 6 which gives ratios for the males and females of mixed ancestry shows that the percentage of lung tumors at the different age periods was consistently higher for the males than for the females. For the males of all ages combined it was 50 per cent compared with 26 per cent for the females. In Text-fig. 3 we see that also in the inbred Strain 1194 the males have a higher lung tumor rate (13 per cent) than do the females (4 per cent) and in the inbred Bagg strain 35 per

cent of the females had lung tumors whereas 45 per cent of the males developed them. In the larger group of 2300 individuals from a variety of sources the rate of incidence was 16 per cent for the females and somewhat greater, 22 per cent, for the males. Whatever factors may explain the high per cent of tumors among the males in certain instances in our stock (it was frequently 2 or more times as high as among the females) sex certainly is not nearly so potent a factor in determining the occurrence of tumors in the lung as it is in affecting tumors of the mammary gland, which are restricted almost entirely to the female sex, having been rarely reported in males.

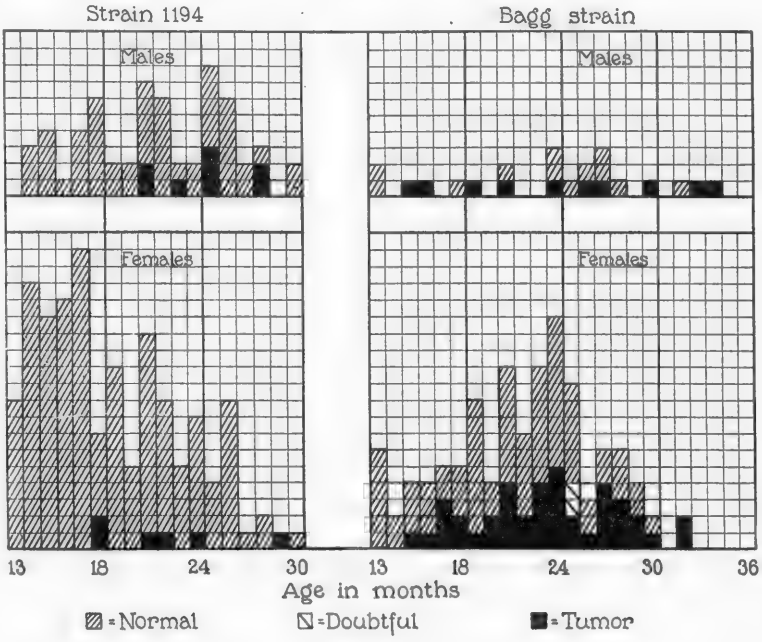
2. Rates of Incidence of Spontaneous Tumors of the Lung in Two Inbred Strains of Mice.—Different strains of mice show differing rates of incidence of tumors of the lung but two inbred strains have been discovered which present a marked contrast to each other.

Strain 1194 is one of the Lathrop stocks and was derived originally from a cross between a male of one tumor strain and 2 females from another strain. Their descendants were inbred probably rather closely for four generations before they were acquired by this laboratory. One pair of mice in the fifth generation gave rise to the present strain and their offspring have been bred brother by sister (or rarely, cousin by cousin) since that time. These data include individuals from the sixth to the fifteenth generation. Most of the mice are black agouti like the wild house mouse though a few are pink-eyed or brown agouti. The stock has a low rate of incidence for tumors of the lung but a high rate for tumors of the mammary gland. Growths may appear in the mammary gland when the mouse is but 5 months of age but in this strain they usually appear at about 12 months. 65 per cent of the females that have been bred and live to be 6 months old and 20 per cent of those that have not been bred have mammary gland tumors. Tumors of the lung are noted only at autopsy. Though they may appear when the mouse is but 8 months old in the general stock, in this strain the youngest age at which a lung tumor was found was 18 months. In all, there were but 14 animals with lung tumor out of a total of 208 mice that lived to be a year old—a rate of 6.7 per cent.

For comparison with Strain 1194 we may cite the Bagg strain of albinos. This stock in contrast with Strain 1194 has a high incidence of lung tumors and a lower incidence of mammary gland tumors. It is impossible to say whether or not any significance attaches to the fact that each strain gives a high and a low tumor rate but in the opposite type of tumor. The data cover a group of 30 or 40 mice from a stock which had been rather closely inbred for several years previous to their acquisition by us and also two generations from a half dozen pairs which were inbred brother by sister. Among females which have been bred,

mammary gland tumors occur in about 27 per cent and in 4 per cent of females over 6 months that have not been bred; while in a total of 135 individuals over 12 months tumors of the lung are found in 37.04 per cent.

Since age and sex in regard to tumor production are of interest, the distribution of the individuals in the two strains is presented graphically in Text-fig. 2 and a summary of the ratios arranged in age periods of 6 months each is given in Text-fig. 3. The mortality of females under



TEXT-FIG. 2.

18 months of age was greater in Strain 1194 than in the Bagg strain and tumors did not appear until the 18th month in the first strain as compared with the 15th month in the Bagg albinos. Although some of the totals are small, in each of the age periods for both males and females the ratio of tumor to non-tumor individuals is higher in the Bagg strain than it is in Strain 1194. Among the Bagg males of all ages over 12 months there were 10 mice with lung tumors and 12 non-

tumor mice (45 per cent of lung tumors), whereas in Strain 1194 there were 8 males with tumors and 52 non-tumor males (13 per cent). Among the females, the ratio in the Bagg strain was 40 tumor mice to 73 non-tumor mice (35 per cent) and in Strain 1194 there were 6 tumor mice to 142 non-tumor mice (4 per cent). Combining individuals of both sexes and all ages the rate of incidence of tumors of the lung in the Bagg strain was 37.04 per cent ± 2.80 in a total of 135 mice and in Strain 1194 it was only 6.73 per cent ± 1.17 in a total of 208 mice. The difference between the tumor rates of the two strains is 30.31 ± 3.04. Since the difference is almost 10 times the probable error the

Females

Strain	Age, 13-18 mos.		19-24 mos.		25-36 mos.		Total		
	No tumor	Tumor	No tumor	Tumor	No tumor	Tumor	No tumor	Tumor	Per cent
Bagg strain	19	7	38	18	16	15	73	40	35
Strain 1194	77	2	48	3	17	1	142	6	4

Males

Strain	Age, 13-18 mos.		19-24 mos.		25-36 mos.		Total		
	No tumor	Tumor	No tumor	Tumor	No tumor	Tumor	No tumor	Tumor	Per cent
Bagg strain	3	2	3	3	6	5	12	10	45
Strain 1194	18	0	18	3	16	5	52	8	13

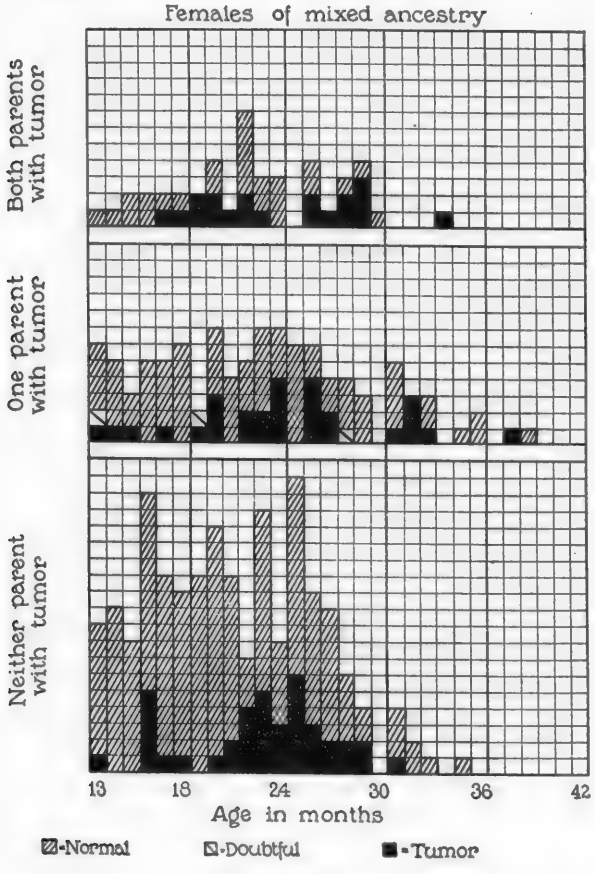
TEXT-FIG. 3.

chances are enormously against its being due to fluctuations in sampling. We may conclude that it is undoubtedly significant.[7]

3. Comparison of Rates of Incidence among the Offspring of Parents Which Have and of Parents Which Have Not Developed Primary Tumors of the Lung.—Records are available of a very mixed population derived from crosses between mice from a variety of sources. The offspring from these matings may be grouped in three classes: mice (1) from parents in which no lung tumors were found, (2) from parents one of which developed a lung tumor, and (3) from parents both of which

[7] The probable error of the percentages was calculated by the method given by Haldane (Haldane, J. B. S., *J. Genetics*, 1919, viii, 291).

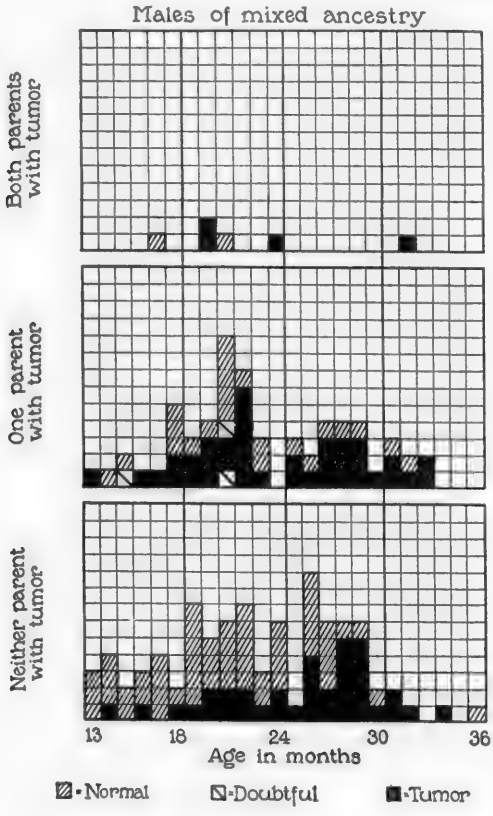
developed lung tumors. In certain cases the autopsy record of one of the parents was not available for reasons already stated. The offspring of such a parent were included in the data, the parent being classed as a non-tumor mouse. This procedure is justified because the



TEXT-FIG. 4.

data include also mice from parents which died younger than the earliest age at which tumors have ever been seen and because at best the classification is inexact. Probably many mice never produce tumors

although they have an inherited capacity to do so. Such individuals must be classed as non-tumor mice whereas genetically they are tumor mice. Class 1, above, which comprises mice from non-tumor parents probably includes individuals which should go in Class 2 or



TEXT-FIG. 5.

3, and Class 2 probably includes mice of which both parents were genetically speaking tumor mice and should have been put in Class 3. The males and females are grouped separately and the age distribution of the three classes for each sex is shown in Text-figs. 4 and 5. The distribution of deaths covers about the same range for the three

Females of mixed ancestry

Type of mating	Age, 13-18 mos.			19-24 mos.			25-30 mos.			31-36 mos.			37-40 mos.			Total		
	No tumor	Tumor	Per cent tumors	No tumor	Tumor	Per cent tumors	No tumor	Tumor	Per cent tumors	No tumor	Tumor	Per cent tumors	No tumor	Tumor	Per cent tumors	No tumor	Tumor	Per cent tumors
0×0	59	8	12	55	15	21	34	15	31	7	1	13	0	0	0	155	39	20
T×0	25	4	14	19	12	39	16	6	27	9	5	36	1	1	50	70	28	29
T×T	8	2	20	12	8	40	5	8	62	0	1	100	0	0	0	25	19	43

Males of mixed ancestry

Type of mating	Age, 13-18 mos.			19-24 mos.			25-30 mos.			31-36 mos.			37-40 mos.			Total		
	No tumor	Tumor	Per cent tumors	No tumor	Tumor	Per cent tumors	No tumor	Tumor	Per cent tumors	No tumor	Tumor	Per cent tumors	No tumor	Tumor	Per cent tumors	No tumor	Tumor	Per cent tumors
0×0	15	3	17	24	10	29	12	18	60	1	4	80	0	0	0	52	35	40
T×0	5	5	50	10	14	58	5	13	72	2	5	71	0	0	0	22	37	63
T×T	1	0	0	1	3	75	0	0	0	0	1	100	0	0	0	2	4	67

0×0 = neither parent with tumor
T×0 = one parent with tumor
T×T = both parents with tumor

TEXT-FIG. 6.

classes when their respective age periods are compared. The occurrence of the doubtful cases is indicated on the graphs but they are omitted from the summary of the ratios which is given in Text-fig. 6.

The data for both sexes show that the rate of incidence of tumors of the lung is higher among the offspring when the parents are known to have developed lung tumors than when the parents were tumor-free.

Among the females of all ages in the first class (from tumor-free parents) there were 155 non-tumor mice to 39 tumor mice, or 20 per cent; in the second class (in which one of the parents developed a lung tumor) there were 70 non-tumor to 28 tumor mice, or 29 per cent; in the third class (in which both parents had tumors) the percentage of tumors increased still more as there were 25 non-tumor and 19 tumor mice, or 43 per cent. Among the males, in the first class there were 52 non-tumor and 35 tumor mice, or 40 per cent and in the second class, 22 non-tumor mice to 37 with tumors, or 63 per cent. There were but 6 individuals in the third class but 4 of them developed tumors. Examining the ratios for the different age periods we find evidence of the same increase in tumor rate in the groups which have a tumor ancestry over groups with a non-tumor parentage. Among the females between 12 and 18 months of age the rate increases slightly from 12 per cent in the first class to 14 per cent in the second. In the third class there are but 10 mice but 2 of them (20 per cent) had lung tumors. Females between 19 and 24 months in the first class had a tumor rate of 21 per cent; in the second class 39 per cent and in the third class 40 per cent. In the age period between 25 and 30 months is found one of the two instances in which the tumor rate for mice in the second class fell below that of the first class. From parents free from lung tumors there were 34 non-tumor and 15 lung tumor mice, or 31 per cent. The number of offspring in cases in which one of the parents had a lung tumor was small. There were 16 mice without lung tumors and 6 mice which developed them, or 27 per cent. From crosses between 2 parents with lung tumor there were fewer individuals (5) without lung tumor than there were with lung tumor (8). The numbers in the age period from 31 to 36 months are small but the percentage of tumors increases from the first to the third class.

Among the males, the numbers in the several age periods are small

but the percentages are consistently higher in the second and third classes except in the age period of 31 to 36 months. The largest groups are found between 19 and 24 months of age. From parents free from tumor (Class 1) there were 24 non-tumor to 10 tumor mice (29 per cent); from parents one of which had a tumor (Class 2) there were 10 non-tumor to 14 tumor mice (58 per cent); there were 4 mice from parents both of which had tumor and 3 out of the 4 were tumor mice.

The two inbred strains—the Bagg albinos and Strain 1194—have not been included in the above figures. The distribution of the indi-

Females of Strain 1194

Type of mating	Age, 13-18 mos.		19-24 mos.		25-30 mos.		Total	
	No tumor	Tumor	No tumor	Tumor	No tumor	Tumor	No tumor	Tumor
0 × 0	68	2	40	1	15	1	123	4
T × 0	6	0	6	1	0	0	12	1

Males of Strain 1194

Type of mating	Age, 13-18 mos.		19-24 mos.		25-30 mos.		Total	
	No tumor	Tumor	No tumor	Tumor	No tumor	Tumor	No tumor	Tumor
0 × 0	13	0	18	3	16	4	47	7
T × 0	5	0	0	0	0	0	5	0

0 × 0 total for males and females = 170 no tumor to 11 tumor
T × 0 " " " " " = 17 " " = 1 "

0 × 0 = neither parent with tumor
T × 0 = one " " "

TEXT-FIG. 7.

viduals according to age, sex, and class is shown in Text-figs. 7 and 8. In Strain 1194 the majority of the matings were between parents in which no tumor was found. Of their offspring (males and females) there were 170 which did not develop lung tumors and 11 which did develop tumors, giving a tumor rate of 6 per cent. Of the 18 offspring from matings between tumor and non-tumor parents only one developed a growth in the lung. There were no matings between 2 tumor mice. In the Bagg strain, from non-tumor parents there were 2 non-tumor mice and one individual with a tumor. From crosses in which one parent had a tumor the ratio was 15 non-tumor and 15 tumor mice and

from matings in which both parents had lung tumors the ratio was 5 to 6. In these strains little difference is seen in the ratios from the various types of crosses.

If the available data are combined it is found that among the offspring of both sexes from non-tumor parentage there was a total of 465 mice of which 86 developed lung tumors (19 per cent); from parents one of which had tumor, there were 205 mice of which 81 developed tumors (40 per cent) and from doubly cancerous parentage

Females of Bagg strain

Type of mating	Age, 13-18 mos.		19-24 mos.		25-30 mos.		Total	
	No tumor	Tumor	No tumor	Tumor	No tumor	Tumor	No tumor	Tumor
0 × 0	0	0	0	1	0	0	0	1
T × 0	2	1	6	4	3	4	11	9
T × T	0	2	0	0	5	4	5	6

Males of Bagg strain

Type of mating	Age, 13-18 mos.		19-24 mos.		25-30 mos.		Total	
	No tumor	Tumor	No tumor	Tumor	No tumor	Tumor	No tumor	Tumor
0 × 0	0	0	1	0	1	0	2	0
T × 0	0	1	1	2	3	3	4	6
T × T	0	0	0	0	0	0	0	0

0 × 0 total for males and females = 2 no tumor to 1 tumor
T × 0 " " " " " = 15 " " " 15 "
T × T " " " " " = 5 " " " 6 "

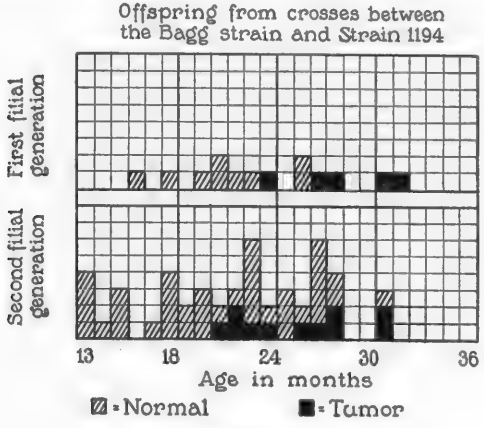
0 × 0 = neither parent with tumor
T × 0 = one parent with tumor
T × T = both parents with tumor

TEXT-FIG. 8.

there were 61 progeny of which 29 had tumors (48 per cent). These totals and the figures given in the separate analyses show that mice are more likely to have lung tumors when one of their parents had a lung tumor than if neither parent did and this likelihood is increased still further when both parents have tumors.

4. Crosses between Strains Having a High and a Low Incidence of Tumors of the Lung.—There were a few matings between males from the Bagg strain which has a lung tumor incidence of 37.0 per cent and females from Strain 1194 in which the lung tumor incidence is 6.7

per cent. Only one of the 2 males used developed a tumor. None of the females had growths in the lung. Of the 14 members of the first filial generation which lived to be at least 16 months old, 7 sons were free from lung tumors while 5 developed them. Neither of the 2 daughters had lung tumor though one lived to be 16 and the other 18 months old. These individuals and several more which died before tumor age were inbred to produce the second filial generation. The males of this second generation were used in another experiment since their value for the lung tumor record was at that time unknown. Of the females 49 lived to be more than 12 months old and of them 11 (22 per cent) developed lung tumors. The age distributions of the



TEXT-FIG. 9.

first and second filial generations are represented in Text-fig. 9. In the first filial generation most of the non-tumor mice were younger than the individuals with tumor. The appearance of tumors in the first generation when tumor-free individuals from a low tumor stock were crossed with mice from a high tumor stock suggests that tumor susceptibility is dominant.

SUMMARY AND DISCUSSION.

1. The occurrence of tumors in the lung in mice is dependent to a certain extent upon the age of the individual. No tumors were found

in the lungs of mice less than 8 months old. They occurred with greatest frequency in mice of about 24 months or older. Mice may live to be more than 3 years old without developing growths in the lung. These facts show that the development of tumors of the lung, if hereditary, is a variable character. An individual, genetically a tumor mouse, may live to a great age without showing a tumor if the requisite environmental stimulus (external or internal) is lacking.

Sex if effective at all has a comparatively slight influence upon the incidence of lung tumors.

2. Two strains of mice were studied which exhibit differences in their rates of incidence of lung tumors that are large enough to be significant. The conflict in the evidence from different laboratories as to whether or not tumors of the lung are the commonest type found in mice, is probably to be explained on the basis of a differing hereditary tendency to such growths in differing stocks.

3. Data from a number of sources indicate that offspring from parents free from lung tumors have a lower rate of lung tumor incidence than offspring from parents one of which had a tumor. If both parents had lung tumors the rate of tumor incidence among their offspring is increased still further.

4. In crosses between mice from strains which have high and low rates of incidence of lung tumor, tumors appeared in about half of the individuals of the first generation and in about one-quarter of the second generation. If the character responsible for the development of the growths is recessive it should not be found in the first filial generation unless both parents are carrying it. There is no proof that the female parents did not carry it but since they were taken from a strain in which the incidence of the growths was but 6.7 per cent the chances seem good that they were free from it. This suggests that the character determining the incidence of pulmonary tumors may be a dominant one.

A dominant character is not expected to appear among the offspring from parents neither of which has shown the character. The numerous instances which have been tabulated in this paper of mice with lung tumors among the offspring of parents free from lung tumors, must be explained on the assumption that tumor susceptibility is not

only dominant but variable and that some of the parents which did not actually develop tumors were genetically tumor mice and had the capacity for developing tumors although it was not brought out. As we have already concluded on the basis of the relationship between age and tumor incidence that susceptibility to the development of lung tumors is a variable character our explanation of the occurrence of tumor mice derived from tumor-free parents is justifiable.

The existence of strains of mice with rates of incidence of lung tumors that differ as widely as do the two that we have studied, the relatively high incidence of pulmonary growths among mice of tumor parentage as compared with mice from non-tumor parents, and the fact that females from a strain in which pulmonary tumors are rare when crossed with individuals from a strain in which they are frequent give a fairly high rate of incidence of the growths among the first and second filial generations,—all these facts indicate that susceptibility to the development of tumors in the lung is an inherited character.

EFFECTS OF INSULIN ON THE WEIGHT OF THE RABBIT'S SUPRARENAL GLANDS.

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According to Riddle and his coworkers (1) insulin administered to pigeons usually causes suprarenal enlargement. They gave the insulin in single large doses or repeated smaller doses, and measured the enlargement by weighing the glands. In order to obtain information as to the effects on mammals the present study was conducted. Daily doses of insulin covering periods of several months were given to rabbits. The adrenals were carefully weighed on laboratory balances at the completion of the experiments. The amount of insulin injected was 0.7 "U" unit per kilo of body weight. The gross weights according to Chart 1 did not indicate hypertrophy.

The control series was treated in the same manner as the insulin-treated series with the same amount of normal salt solution. The animals were given a diet of oats and hay (2, 3).

Boycott and Kellaway (4) have concluded that the average total weight of both adrenals for full grown rabbits is 650 mg. The average weight per kilo for both glands is 250 mg. Bager (5) gave 599 mg. as the average total weight, and 246 mg. as the average weight per kilo. He also stated that rabbits above 2312 gm. in weight have no relative increase in the weights of the adrenals. Other workers have given different results. As the number of rabbits examined by Bager and Boycott was large their figures are regarded as accurate.

The control series has greater weights than the insulin-treated series. The average for the control series is slightly larger for the absolute weight, and normal for the weight per kilo. The average weight per kilo for the insulin-treated rabbits is slightly below the normal, and close to normal for the absolute weight. There is no relative decrease

or increase of the weights covering long periods of time. The maximum weights in both series are during the 4th and 8th months in the insulin-treated rabbits, and the 5th month in the control rabbits.

Frozen sections of the adrenals were stained with Sudan III and counterstained with hematoxylin and eosin, in order to observe the lipoids. By means of this qualitative method it was found that there was no appreciable diminution of lipoids in the cortices except a slight decrease in No. 7, in which it could be accounted for by the infection.

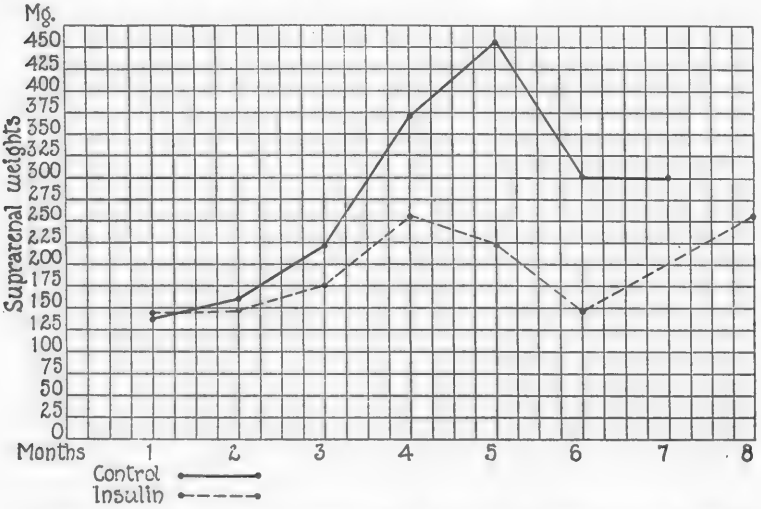

CHART 1. The lower curve represents the weights of the suprarenals of insulin-treated rabbits during 1 to 8 months inclusive. The upper curve represents the weights in the control series.

Anisotropic lipoids could not be demonstrated in the adrenals of rabbits by the usual frozen section method. They could be seen by the method of Albrecht and Weltmann (6). This consists of adding methyl alcohol to the frozen sections, and then a saturated solution of green soap in methyl alcohol. They are then heated, cooled, and examined through the Nicol prism. Anisotropic lipoids are found in abundance in all of the adrenals studied.

There were no noteworthy microscopic pathological changes with the hematoxylin and eosin stain.

Because there were no striking differences in weights and microscopic changes of the insulin-treated rabbits, no further studies were made. Stewart and Rogoff (7) have found no differences in the behaviour of adrenalectomized rabbits treated with insulin.

TABLE I.

Rabbit No.	Weights of adrenals.		Time.	Weight when killed.
	Per kilo.	Total absolute weights.		
	mg.	*mg.*	*mos.*	*gm.*
Insulin-treated.				
1♂	145	380	1	2620
2♀	147	300	2	2030
3♂	176	530	3	3000 (Abscesses 1 mo.)
4♂	253	760	4	3000
5♂	221	955	5	4320 (Huge abscess, 2 wks. duration.)
6♀	141	610	6	4300
7♂	264	818	8	3100
♂ + ♀	178	622		
♂	212	688	1–8	
♀	144	455		
Controls.				
8♂	140	417	1	2970
9♀	155	560	2	3600
10♀	221	730	3	3300
11♀	369	1125	4	3050
12♂	464	1532	5	3300
13♀	294	940	6	3200
14♀	188	600	6	3200
15♀	293	1200	7	4100
♂ + ♀	266	888		
♂	302	975	1–7	
♀	254	859		

SUMMARY.

There is no gross hypertrophy of the adrenals in rabbits after long continued, daily doses of insulin.

My thanks are due to Dr. H. T. Karsner for his criticism of this work.

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THE IMMUNITY PRODUCED BY THE GROWTH OF TETANUS BACILLI IN THE DIGESTIVE TRACT.

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(Received for publication, November 6, 1925.)

In a previous paper (1) we pointed out that while tetanus spores can be found in the stools of about a third of the population in Peking, so far as we could determine tetanus as a disease is relatively rare. Upon examining the sera of carriers we found that an appreciable amount of antitoxin was present and our conclusion was that carriers must be relatively immune to tetanus. We felt, however, that the conclusion should be put to experimental test, and this and the following papers are the results of these tests. We early learned that in testing the immunity of carriers we had to consider the serological types of the bacillus as first described by Tulloch (2) and confirmed by one of the writers (3). Our results show that immunity is specific for the serological type of tetanus bacilli fed or injected and, much to our surprise, that the antitoxin content of the serum does not indicate the degree of resistance to infection.

As experimental animals we have used guinea pigs throughout our work for, as we have already reported (4), it is relatively easy to establish tetanus bacilli in their digestive tracts. When once established the sera of these carriers soon show agglutinins for the type fed while antitoxin appears more slowly. After some months however, this latter antibody can be found in relatively large amounts. Tulloch (2) has shown that the antitoxin produced by the injection of toxin formed by one type of bacilli will neutralize the toxins of all the other types. In other words antitoxin is not specific for type. Our findings agree with this work for, using the toxin formed by Type V bacilli, we find that the sera of animals fed the other types will have approximately the same neutralizing power as the sera from the animals fed Type V. If antitoxin were specific for type we would expect that the

sera of animals fed Type V would show a higher antitoxin content than those fed other types.

In contrast to much of the experimental work that has been done on tetanus we have not tested the immunity of our animals by the injection of toxin but have sought to produce an infection by the injection of spores free from this substance. As is well known spores alone will not produce an infection but some irritating substance must be introduced at the same time in order that they may germinate. Kitasato (5) used for this purpose a splinter of wood, Tulloch (2) saponin, and Bullock and Cramer (6) used $CaCl_2$. We tried all of these agents and were not satisfied with them. Infection and death appear so soon after the inoculation that the test of immunity is a very severe one. We wanted to produce a disease which would as nearly as possible resemble that in man in having an incubation period of several days and as long a duration as possible. After considerable search we selected the method given below. It is not ideal in that after it once appears the disease progresses rapidly but it is more satisfactory than any other we have tried.

Methods.

We have used the same methods in a number of studies and shall give them here in some detail.

Aleuronat is the irritating substance that we have used to enable the tetanus spore to germinate and grow. We make it up with starch as for the production of an exudate except that we use a 3 per cent instead of a 5 per cent suspension as the latter is too thick to be injected with ease. The factor that has probably helped us most in securing a long incubation period is that we have used a small number of spores (about 1000). We prepare our spore suspension as follows:

Bouillon cultures under vaseline of known types of tetanus bacilli that have been incubated 8 days and then placed in the refrigerator are the source of the spores. When wanted, about 10 cc. of culture is centrifugalized and the sediment is suspended in salt solution. This suspension is heated for 20 minutes at 70° and after cooling known dilutions are made in sterile salt solution. From the higher dilutions 1 cc. amounts are transferred from each to labeled sterile 1 inch test-tubes, duplicate tubes being made for each dilution. To each tube is now added 5 cc. of clear liver digest agar (7) that has been melted and cooled to 45°. The tubes are kept in water at 45° until the agar has been added to all, when they are taken out one at a time and Esmarch roll cultures made by rotating the tubes on a block of ice. The agar containing the spores is thus deposited as a thin film

on the sides of the tubes. Sterile melted agar at 45° is now carefully added to each of the tubes until it comes well above the level of the films, care being taken not to detach the latter from the sides of the tubes. After immersion in ice water until the agar has solidified the tubes are incubated. Usually in 24 hours but sometimes not until a day later, the spores that have been deposited on the sides of the tubes have developed and colonies large enough to count have been formed. Suitable tubes are counted and the number of spores per cc. of the original suspension is calculated. This method of determining the number of spores has proven quite accurate, it is easier than the direct microscopical count, and it has the additional advantage that it gives us the number of viable spores only. It seems to us that this might be a useful method of studying colony formation of anaerobes as the growth is just under the surface of the glass.

Experiments have shown that tetanus spores are not easily destroyed in salt solution as counts made after the suspensions have been stored 2 days in the refrigerator agree quite closely with those made on the day of the preparation of the suspension. Our practice therefore has been to place the suspensions in the refrigerator until counts have been made and the number of spores per cc. has been determined. The original suspension is then diluted until it theoretically contains 10,000 spores per cc. and from a 1:1000 dilution of this two anaerobic roll cultures as a check on the first count are made. To a flask containing 90 cc. of freshly sterilized and warm 3 per cent aleuronat plus 3 per cent starch in distilled water we add 10 cc. of the suspension supposed to contain 10,000 spores per cc. After mixing thoroughly, the flask containing the mixture is placed in a jar from which the air is then partially exhausted in order to remove the air bubbles from the aleuronat. This mixture which should contain, and as counts have shown does contain, approximately 1000 spores per cc. is then injected into the muscles of the hind legs of our animals, by means of a 1 cc. syringe and a No. 18 needle.

As a matter of fact it makes little difference whether 1000 or 10,000 spores are injected as shown in Table I. The results indicate that with aleuronat the injection of 1000 spores invariably produces tetanus while alone 50,000 spores failed to induce infection. In another experiment two guinea pigs that were each given 1 cc. of aleuronat suspension containing 160 Type V spores both developed tetanus on the 7th and 8th days respectively. The former died on the 12th day after the injection while the latter recovered. In all our experiments we keep animals that fail to develop tetanus under observation for 2 months before they are discarded. This is done because, as will be noted later, when we were using another method some of our animals developed tetanus a month after inoculation. With the method here described we have not had this difficulty.

An examination of the results given in this and the papers that are to follow will show that our control animals always developed tetanus and that symptoms usually appeared on the 5th though sometimes on the 4th day. In all these animals death followed on the 7th to the 10th

TABLE I.

Test of Ability of 1 Cc. of 3 Per Cent Aleuronat to Produce Tetanus When Mixed with Different Types of Tetanus Spores.

Type of spores used.	Spores suspended in aleuronat.				Spores suspended in salt solution.			
	No. of spores injected.	Guinea pig No.	Weight.	Result.	No. of spores injected.	Guinea pig No.	Weight.	Result.
			gm.				*gm.*	
I	10,000	1	320	4th day—local tetanus. 7th " death.	50,000	5	300	No tetanus.
"	"	2	230	2nd " local tetanus. 6th " death.	"	6	330	" "
"	1,000	3	320	5th " local tetanus. 9th " death.	10,000	7	?	" " 2nd day—death; cause?
"	"	4	280	5th " local tetanus. 8th " death.	"	8	310	No tetanus.
II	10,000	9	310	5th " local tetanus. 7th " death.	50,000	13	290	" "
"	"	10	250	5th " local tetanus. 7th " death.	"	14	390	" "
"	1,000	11	220	5th " local tetanus. 7th " death.	10,000	15	420	" "
"	"	12	250	5th " local tetanus. 7th " death.	"	16	220	" "
III	10,000	17	280	4th " local tetanus. 7th " death.	50,000	21	280	" "
"	"	18	250	4th " local tetanus. 7th " death.	"	22	520	" "
"	1,000	19	410	5th " local tetanus. 8th " death.	10,000	23	390	" "

III	1,000	20	410	5th day—local tetanus. 7th " death.	10,000	24	290	No tetanus.
V	10,000	25	280	5th " local tetanus. 7th " death.	50,000	29	240	" "
"	"	26	330	5th " local tetanus. 7th " death.	"	30	380	" "
"	1,000	27	250	5th " local tetanus. 7th " death.	10,000	31	410	" "
"	"	28	290	5th " local tetanus. 7th " death.	"	32	290	" "

TABLE II.

Immunity of Carriers to Type III Spores.

Guinea pig.		Type of spores fed on Aug. 28, Sept. 3, Sept. 20, 1924.	Tests on serum.						Results of intramuscular injection of 1000 Type III spores suspended in 1 cc. of 3 per cent aleuronat emulsion. Feb. 20, 1925.
No.	Weight at time of inoculation.		Date of bleeding.	No. of M.L.D. of toxin neutralized by 0.25 cc. of serum.	Agglutination titre for type. I	II	III	V	
	gm.								
33	920	III	Feb. 10	50	20—*	20—	160	20—	No tetanus during next 60 days.
34	610	"	" "	50	20—	20—	320	20—	Found dead on 10th day. No tetanus. Abscess at site of inoculation.
35	820	I	" "	50	160	20—	20—	20—	Generalized tetanus 6th day. Death 7th day.
36	910	"	" "	75	160	20—	20—	20—	" "
37	640	V	" "	50	20—	20—	20—	160	Local tetanus on 5th day. Death on 6th day.
38	600	"	" "	75	20—	20—	20—	160	" "
39	600	"	" "	50	20—	20—	20—	320	" "
40	480	Controls.	" 9	2—†	Not tested.				" "
41	480	"	" "	2—	" "				" "
42	500	"	" "	2—	" "				Local tetanus on 5th day Death on 7th day.
43	520	"	" "	2—	" "				Local tetanus on 5th day. Death on 6th day.

* The figures are the highest dilution in which agglutination was observed. When followed by a minus no agglutination was present in this the lowest dilution used.

† 2— means that 0.25 cc. serum failed to neutralize 2 M.L.D. of toxin.

TABLE III.

Immunity of Carriers to Type I Spores.

Guinea pig.		Type of spores fed on Aug. 28, Sept. 3, Sept. 20, 1924.	Tests on serum.						Results of intramuscular injection of 1000 Type I spores suspended in 1 cc. of 3 per cent aleuronat emulsion. Mar. 13, 1925.
No.	Weight at time of inoculation.		Date of bleeding.	No of M.L.D. of toxin neutralized by 0.25 cc. of serum.	Agglutination titre for type.				
					I	II	III	V	
	gm.								
44	680	I	Feb. 10	25	320	20—	20—	20—	No tetanus during next 60 days.
45	630	"	" "	10	160	20—	20—	20—	" " " " " "
46	1010	III	" "	50	20—	20—	160—	20—	Local tetanus 5th day. Death 7th day.
47	725	"	" "	25	20—	20—	160—	20—	" " " " " " "
48	800	V	" "	75	20—	20—	20—	320—	" " " " " " "
49	695	"	" "	75	20—	20—	20—	320—	" " " " " 6th "
50	390	Controls.	Mar. 2	2—	Not tested.				" " " " " " "
51	400	"	" "	2—	" "				" " " " " " "
52	435	"	" "	2—	" "				" " " " " 7th "
53	440	"	" "	2—	" "				" " " " " " "

TABLE IV.

Immunity of Carriers to Type V Spores.

Guinea pig.		Type of spores fed on Aug. 28, Sept. 3, Sept. 20, 1924.	Tests on serum.						Results of intramuscular injection of 1000 Type V spores suspended in 1 cc. of 3 per cent aleuronat emulsion. Mar. 20, 1925.
No.	Weight at time of inoculation.		Date of bleeding.	No. of M.L.D. of toxin neutralized by 0.25 cc. of serum.	Agglutination titre for type.				
					I	II	III	V	
	gm.								
54	610	I	Feb. 10	50	320	20—	20—	20—	4th day—local tetanus; 5th day—general tetanus; 6th day—death.
55	720	"	" "	50	160	20—	20—	20—	4th day—general tetanus; 5th day—death.
56	750	III	" "	75	20—	20—	160	20—	4th day—local tetanus; 6th day—general tetanus; 7th day—death.
57	760	"	" "	25	20—	20—	160	20—	" "
58	760	V	" "	50	20—	20—	20—	160	No tetanus during next 60 days.
59	720	"	" "	50	20—	20—	20—	160	" " " " " "
60	430	Controls.	Mar. 10	2—	Not tested.				4th day—general tetanus; 6th day—death.
61	430	"	" "	2—	" "				" " " " " " "
62	420	"	" "	2—	" "				4th day—local tetanus; 5th day general tetanus; 7th day—death.
63	440	"	" "	2—	" "				4th day—death.

day after inoculation. In order to secure such uniform results it is necessary to use for controls guinea pigs with stools that, on culture, show no tetanus-like bacilli and with sera free from antitoxin.

For the cultural test two or three fecal pellets from each animal are suspended in approximately 3 cc. of sterile salt solution and heated 20 minutes at 75°, after which the whole suspension is transferred to a deep tube of freshly heated cooked meat medium. If present, tetanus bacilli bearing the typical round terminal spores are found in the greatest numbers in this medium after 5 days incubation. Films are therefore made from the fecal cultures on the 4th and 5th days of incubation and if tetanus-like bacilli are found the guinea pig in question is discarded. About one-third of our guinea pigs carry tetanus bacilli in their digestive tracts and therefore are not satisfactory for this work. Those animals with negative feces for tetanus-like bacilli are bled from the heart and their sera tested as follows: 0.5 cc. of serum, 0.5 cc. of salt solution, and 1 cc. of a solution of carefully standardized dried toxin diluted so that it contains four minimal lethal doses for a hamster per cc. are mixed in a small test-tube and allowed to stand half an hour at room temperature. The hamster (*Cricetulus griseus*, M.-Eds.) has been used as a test animal in this work because it is the cheapest laboratory animal we have and it reacts quite uniformly to tetanus toxin. Each animal is injected subcutaneously with 1 cc. of the above mixture and is kept under observation for 4 days. If at the end of this time the hamster is alive, *i.e.* if 0.25 cc. of serum has neutralized 2 M.L.D. of toxin, the guinea pig that supplied this serum is not used for our work. Very rarely do we find antitoxin in the blood of a guinea pig which we have passed as having no tetanus-like bacilli in the stool. This test is therefore used as an additional precaution and a check on the cultural method.

Having developed methods for producing in the guinea pig, by the injection of spores, an infection that had an incubation period of approximately 4 days and that always ended fatally, we proceeded to test the immunity of animals that had been carrying known types of tetanus bacilli for a known period.

EXPERIMENTS.

Immunity Following the Feeding of One Type of Tetanus Spores.

In August, 1924, a number of guinea pigs were selected and, after it had been shown that their feces were negative for tetanus-like bacilli and their sera free from antitoxin and agglutinins for the various types of tetanus bacilli, they were divided into three equal lots. On 3 different days these animals were fed the heated washed sediment from old bouillon cultures of tetanus bacilli, one lot being given Type I, another Type III, and the third Type V. The cultures of Types I and III used for the feeding are Tulloch's key strains while the Type V

is a culture we had isolated. The feeding was done by withholding food from the animals for a day and then placing the spore material on their usual food. Some 6 months later, at dates which will be found in the tables, these animals were bled and their sera tested for antitoxin as well as for agglutinins for the various types of tetanus bacilli. The results of these tests which are given in Tables II to IV confirmed our previous report in that only agglutinins for the type bacillus fed were found in the sera and that antitoxin was present in all. When 0.25 cc. serum neutralizes 50 M.L.D. of our toxin, tests have shown that the serum contains 0.05 U. S. A. units of antitoxin per cc.

On the dates given in the tables the animals were injected with spores from known types of tetanus cultures, suspended in aleuronat prepared according to the method given above. The results are so clear-cut that little comment is necessary. When injected with a culture other than that fed the animals develop tetanus and die in the same time as the controls, in spite of the fact that their serum shows considerable antitoxin. When injected with the culture fed the animals fail to develop tetanus though in some cases their serum had a lower antitoxin content than did those fed other types. In other words, one culture of tetanus bacillus established in the digestive tract will produce in 6 months an immunity to this culture but not to cultures of other types and the immunity is not dependent upon the antitoxin content of the blood.

After our experiments were nearly completed we realized that we had made a mistake in using for inoculations the same cultures that were used for feeding. The only conclusion we could draw was that the animals were immune to the culture fed whereas we believe that they are immune to all organisms of the serological type present in the digestive tract. Unfortunately at this time we had only a few animals that had carried organisms for a sufficient length of time for immunity to develop but the results of the inoculation given in Table V show that the immunity is specific for the serological type. This point will be brought out more fully in papers which are to follow.

While we have only to a limited extent studied the development of immunity after feeding the results indicate that it comes on rather slowly. 4 months after feeding animals may or may not resist the culture used for the feeding though their serum will at this time show a considerable amount of antitoxin. After carrying one culture for from 6 to 8 months they are immune to this culture but not to cultures

of other types. Whether they would eventually develop an immunity to other types is a question we cannot positively answer though our results all indicate that immunity is strictly limited to the serological type. This too is a point that will be brought out in further papers.

That the immunity obtained by feeding is not absolute was shown in some experiments we made over a year ago. At that time we were producing tetanus by inserting under the skin glass capsules filled with a spore suspension, the capsule being crushed after insertion.

TABLE V.

Test of Immunity of Guinea Pig Carriers of Culture XIX Type V to Other Cultures of Same Type.

Each animal was injected with 1000 spores suspended in aleuronat. May 21, 1925.

Culture used.		Carrier guinea pigs.			Control guinea pigs.		
No.	Source.	No. of M.L.D. of toxin neutralized by 0.25 cc. serum.	Weight.	Result.	No. of M.L.D. of toxin neutralized by 0.25 cc. serum.	Weight.	Result.
			gm.			*gm.*	
190	Feces of soldier.	50	600	No tetanus.	2—	470	Local tetanus 5th day. Death 7th day.
5158	Wound, human tetanus.	50	790	" "	2—	350	" "
T V	Tulloch.	50	670	" "	2—	460	Local tetanus 5th day. Death 8th day.
146	Guinea pig feces.	25	770	" "	2—	480	" "

Our suspension was much more concentrated than the one we have been using in the recent experiments as each capsule contained over a hundred thousand spores. Tetanus developed in the controls and the animals died on the 7th or 8th day. The animals inoculated with types other than that fed also died with tetanus in the same time as the controls. The guinea pigs inoculated with the same culture which they had been fed showed no tetanus when the others were all dead, but from 12 to 25 days after the inoculation they, with two exceptions,

developed a fulminating type of infection and died usually on the day that symptoms were first noted. Our interpretation was that the constant irritation produced by the bits of glass in the subcutaneous tissues enabled the bacilli to grow and that gradually the immune substances were exhausted. Why the animals should show such a rapidly progressing disease we have not been able to explain. The

TABLE VI.

Immunity of Carriers of Several Types of Tetanus Bacilli.

Guinea pig.		Tests on serum.						Date of injection.	Type spores used.	Results of intramuscular injection of 1000 spores suspended in 1 cc. of 3 per cent aleuronat emulsion.
No.	Weight at time of inoculation.	Date of bleeding.	No. of M.L.D. of toxin neutralized by 0.25 cc. of serum.	Agglutination titre for type.						
				I	II	III	V			
	gm.									
64	720	Dec. 2	50	320	320	320	160	Feb. 20	III	Local tetanus; recovery. 5th day—local tetanus; 14th day—death.
65	880	" "	10	320	320	160	160	" "	"	
66	830	" "	50	640	1280	40	320	" "	"	No tetanus.
67	780	" "	50	320	640	80	320	" "	"	" "
68	970	" "	10	160	320	160	640	Mar. 13	I	" "
69	550	" "	25	160	640	160	640	" "	"	" "
70	875	" "	50	320	320	160	320	" "	"	" "
71	780	" "	50	320	160	320	320	" "	"	" "
72	960	Mar. 24	50	320	320	160	320	" 30	V	" "
73	980	" "	25	160	320	160	640	" "	"	" "
74	815	" "	50	320	320	160	160	" "	"	" "
75	930	" "	50	320	160	320	80	" "	"	Local tetanus, 5th day; death, 10th day.

For controls see Tables II to IV.

two guinea pigs that failed to develop the disease had by mistake been placed with a male and were in a late stage of pregnancy at the time they were injected. Living young were born a few days later and the mothers showed no evidence of infection though they were kept under observation for some months. Without further tests we should hesitate to say that pregnancy had raised the resistance to tetanus.

Immunity of Guinea Pigs Fed Several Types of Tetanus Spores.

In contrast to the above experiments in which it has been shown that feeding one type of spore protects against that type only are the results of the tests made on animals that were fed a mixture of types. It will be seen in Table VI that most of the animals were immune to all the types tested and that those that developed tetanus did so later than the controls, indicating that they had a partial immunity. The difference in weight between the animals under test and the controls cannot account for this difference for the results given in the previous tables show that weight plays little part in the resistance to infection.

These results are not strictly comparable to those obtained in the animals fed a single type, as these guinea pigs had been carrying tetanus bacilli in their digestive tracts for a much longer period. They were first fed a single type of spore in June, 1923, and in the spring of 1924 were fed a mixture of spores of all types. When finally inoculated on the dates given in the table care was taken to select for each experiment animals that received at the first feeding a type other than that used for the inoculation.

That more than one type had grown in the digestive tract is indicated by the results of the agglutination tests made with the sera of these animals. Whereas in the previous experiment animals fed a single type showed agglutinins for that type only, here all the types tested were agglutinated by the sera. Antitoxin was found in the sera in approximately the same amounts as in the animals fed a single type.

Resistance of Carrier Guinea Pigs to Tetanus Toxin.

Contrary to our expectation guinea pigs that carry tetanus bacilli in their digestive tracts show little, if any, resistance to tetanus toxin in spite of the fact that their sera contains antitoxin. Our study of this phase of the immunity was not made on animals that had been fed known types of spores but on guinea pigs from our stock whose feces showed tetanus-like bacilli in their stools and antitoxin in their blood.

The data pertaining to the animals as well as the effect of the injec-

tion of toxin are given in Table VII. The intravenous injections were made several days after the subcutaneous injections with the same toxin solution used for the first test. The solution had been kept in the refrigerator in the interval and it seems probable that some of the toxin had been destroyed for it will be noted that 0.00075 mg. injected subcutaneously kills while twice this amount injected intravenously produces no effect.

TABLE VII.

Test of Resistance of Carrier Guinea Pigs to Tetanus Toxin.

Guinea pig.	Result of examination of stool for tetanus-like bacilli.	Amount of toxin neutralized by 0.5 cc. serum.	Tetanus toxin injection.		
Weight.			Amount.	Route.	Result.
gm.		*mg.*	*mg.*		
440	Negative.	0.0006—	0.00075	Subcutaneous.	48 hrs. local tetanus; 6 days 5 hrs. death.
440	"	"	"	"	53 hrs. local tetanus; 5 days 4 hrs. death.
470	"	"	0.0015	"	46 hrs. local tetanus; 72 hrs. death.
440	"	"	"	"	46 hrs. local tetanus; 70 hrs. death.
490	Positive.	0.003	0.003	"	46 hrs. death.
380	"	0.0075	"	"	" " "
460	"	0.003	0.0075	"	33 hrs. general tetanus; 46 hrs. death.
350	"	0.0075	"	"	" "
310	Negative.	0.0006—	0.0015	Intravenous.	No tetanus.
300	Positive.	0.0075	"	"	" "
330	Negative.	0.0006—	0.003	"	40 hrs. death.
340	Positive.	0.0075	"	"	" " "

Our experiments do show that when 0.5 cc. of serum contains enough antitoxin to neutralize the amount of toxin injected, the animals are just as susceptible as those which have no antitoxin.

This phenomenon was first noted by Wladimiroff (8) and has been observed in laboratories where antitoxin is being prepared. We have no explanation other than that commonly given, that it takes some time for toxin and antitoxin to combine and that toxin has a greater affinity for nerve cells than for antitoxin.

The observation has a practical application for if guinea pigs that carry tetanus bacilli were resistant to toxin a considerable error would be introduced in the standardization of antitoxin. While our experiments are not extensive enough to rule out this factor absolutely they indicate that it is not of great importance.

DISCUSSION.

We have shown elsewhere (4) that soon after tetanus bacilli are fed to guinea pigs, agglutinins for the type fed appear in the blood, and that even after 6 months the agglutinins remain specific. Antitoxin appears more slowly but by the end of 6 months it amounts to about 0.05 U. S. A. units per cc. of serum. This is not a high content if one thinks of units but it will neutralize a considerable amount of toxin.

We assumed that this antitoxin would render the animals immune to tetanus bacilli as at the beginning of these experiments we regarded antitoxin as an adequate protecting antibody. The results given in this paper show this assumption not to be justified, for while the guinea pigs are shown to be immune to the type organisms fed they are just as susceptible to organisms of other types as are control animals. *We believe therefore that antitoxin plays little part in acquired immunity to tetanus but that there are other bodies, specific for type, which protect animals against infection.* We shall return to this point in a paper that is to follow on the protective action of sera of animals immunized by the injection of tetanus bacilli.

Guinea pigs can be made immune to more than one type of tetanus bacilli if these types are growing in the digestive tract. While we have never tried to determine how many types of these organisms could be isolated from the stool of a single human, examination of the sera of many individuals in Peking shows that most of them have agglutinins for several types of bacilli. This, together with the many chances for the introduction of tetanus spores into the digestive tract, makes us feel that man must often carry the common types and that he thereby acquires an immunity. Carrying the bacillus does not necessarily make man absolutely immune for we have seen two cases of infection in individuals from whose stools we obtained tetanus bacilli. In one of these cases the organism in the stool was of the

same serological type as that producing the infection. In the other case infection followed a rupture of the intestines and we were not able to recover the bacillus responsible for the tetanus. It is worthy of note that both cases recovered while of the four cases we have seen whose stools contained no tetanus bacilli, three died in spite of intensive treatment with antitoxin. While these findings seem to us suggestive we do not wish to draw conclusions from so few cases, and it will be some time before we can secure enough data to warrant any conclusions as to the protection afforded man by tetanus bacilli in the intestinal canal of man. Incidentally it may be mentioned that we have been unable to detect any ill effects due to the presence of these organisms in the digestive tract. Although the tetanus bacillus is known to form an hemolysin, blood counts have failed to show an anemia in carrier animals, observations out of accord with the statement of Rabinowitsch (9) that marasmus and death resulted from feeding this organism.

SUMMARY AND CONCLUSIONS.

1. A method for the production of tetanus by the injection of a fixed number of spores is described together with the tests made in selecting animals for experimental work.

2. Guinea pigs fed a single serological type of tetanus bacilli will, after 6 months, show considerable amounts of antitoxin in their sera and will manifest immunity to the type fed. To other types they are just as susceptible as are controls.

3. Animals fed several types are immunized to each of these types. It is pointed out in the discussion that the digestive tract of man may carry several types and that he probably reacts in a manner resembling the guinea pig carriers.

4. Guinea pigs that carry tetanus bacilli and have antitoxin in their sera show little if any resistance to tetanus toxin.

5. As there is no relation between the amount of antitoxin in the blood and immunity to tetanus we believe that other bodies, specific for type, must occur and make for the immunity observed.

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STUDIES ON THE BIOLOGY OF STREPTOCOCCUS.

V. Antigenic Relationships between Strains of Streptococcus from Scarlet Fever and Erysipelas.

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Studies of the biologic characteristics of hemolytic streptococci undertaken in recent years, indicate that certain characteristics are peculiar to groups of streptococci associated with certain diseases. The occurrence of hemolytic streptococci in the throat during scarlatina and in erysipelatous lesions has long been established, yet the specificity of these streptococci has been questioned. The observations of Gabritschewsky (1), Moser (2), Gordon (3), Tunnicliff (4), Bliss (5), Dochez and Sherman (6), Dick and Dick (7), and Blake and Trask (8), have shown that the hemolytic streptococcus bears more than a casual relationship to scarlet fever, since strains of streptococci isolated from lesions during the disease have common agglutination and opsonic reactions, and cultures of these strains are capable of stimulating antibodies of prophylactic and therapeutic value. Birkhaug (9, 10), working along similar lines with streptococci isolated from the margin of the inflammation in cases of erysipelas, finds that these strains agglutinate with erysipelas immune sera, and that the immune serum provides passive cutaneous protection against erysipelas strains inoculated intradermally in rabbits.

The basis for the differentiation of these groups of streptococci, which to all appearances possess specific toxic properties in addition to the property of being able to produce pyogenic infection, has been an agglutination reaction common to the strains isolated from a disease, and the capacity of these strains to produce a toxin which can be neutralized by specific antitoxic sera. The agglutination of scarlatinal strains has been studied by Gordon (3), Bliss (5), Stevens and

Dochez (11, 12), Tunnicliff (4), and Williams (13). With the exception of Williams,—who has obtained the lowest percentage of agglutinable strains among cultures from scarlatina, and who finds that numerous strains from streptococcus infections not associated with scarlet fever, agglutinate with scarlatinal antisera,—these authors agree that scarlatinal strains possess common agglutinative characteristics. Eagles (14) and Birkhaug (10) working with both scarlatinal and erysipelas strains have obtained contradictory results in regard to the crossing between the two groups. Eagles has found that scarlatinal antisera are capable of agglutinating erysipelas strains in a somewhat lower titer than scarlatinal strains agglutinate in these sera. Birkhaug has observed no cross-agglutination between the two groups. Since we have been able to isolate strains of scarlatinal streptococci from cases of tonsilitis which presented none of the symptoms of scarlatina aside from the angina, and have found that these strains both agglutinate and produce a toxin, it is probable that a number of scarlatinal strains are included in every group of cultures from ordinary streptococcic infections. On the basis of this observation the agglutination of a percentage of the strains from pyogenic manifestations of streptococcic infection is to be anticipated.

In the articles we have previously published (11, 12), we have considered only scarlatinal streptococci and the differentiation of these strains from pyogenic cocci; the present work has been directed mainly toward the study of cultures from erysipelas.

Materials and Methods.

The methods employed in the agglutination and the absorption of agglutinin are the outgrowth of several years experience with streptococci. The basis for these methods was originated by Dochez, Avery, and Lancefield (15) in 1918, and consists principally in the agglutination of antigens grown in phosphate-buffered broth. Numerous modifications have been made, however, and the methods employed for obtaining proper antigens are best described in detail.

The strains to be agglutinated are first transferred several times in broth culture until they grow readily in this medium. They are then transferred to tubes of phosphate-buffered broth to which two or three cylinders of sterile potato have been added. If these tubes are seeded heavily and the medium has been properly

made, growth is practically uniform after 18 to 20 hours at 37°C. Exceedingly granular strains may be grown at room temperature. The cultures are centrifuged, the supernatant broth poured off, and the sediment is suspended in 20 cc. of neutral distilled water. After shaking, the diffuse suspension is again centrifuged and resuspended in buffered broth to one-fifth the original volume of the culture. The antigens are then refrigerated overnight, and those which show spontaneous agglutination or have granular sediment are rejected. The antigens which appear suitable for agglutination are vigorously shaken and centrifuged at low speed for 1 or 2 minutes. This procedure reduces the titer of the strains with immune sera to from one-half to one-third the titer of unwashed cultures, but the washing is justified by the minimum of difficulty experienced in agglutination. The best reactions are obtained when the dilutions of serum and antigen are agglutinated at 55°C. and refrigerated overnight before reading. The preparation of the immune sera and the methods employed in the dilutions have previously been described (15, 5, 11, 12).

Before the sera were absorbed it was necessary to determine a suitable uniform absorptive dose of antigen. The necessity of employing a uniform dosage is evident, since several sera have been absorbed with numerous strains and the absorbed sera titered for agglutinin with all the strains in the series. With cultures grown as previously described, bacterial counts per cc. do not vary more than one logarithmic exponent. After the uniformity of the growth in these cultures was established, the absorbing dose of each strain in cc. was estimated for the homologous serum. We have found that the washed sediment from 40 cc. of potato broth culture is seldom sufficient to absorb the homologous agglutinin from 5 cc. of 1:20 dilutions in broth, of the sera of the agglutinative titer which we employ. Absorption is complete with 4 cc. in about 70 per cent of instances, and always complete with 2.5 cc. of the diluted sera. This proportion furnishes approximately one and one-half to twice the minimum absorptive dose, and has been employed uniformly in all absorptive tests. The sera are absorbed at 55°C. for 30 minutes and refrigerated until the following morning when the mixtures are centrifuged and diluted.

The cultures have been obtained from various sources. The majority of the scarlatinal strains were obtained in New York City, but cultures are included which were isolated by Dr. Eagles in Toronto, and by Dr. Meleney in Pekin, China. The erysipelas strains have been obtained from the advancing inflammatory margins and from complicating abscesses in cases from Bellevue and Lincoln Hospitals, New York City. Several of Dr. Eagles' strains have been included. The pyogenic strains are from miscellaneous infections occurring in the Presbyterian Hospital.

EXPERIMENTAL.

To determine the specificity of the erysipelas immune sera and strains, six sera were chosen corresponding to the first six erysipelas

TABLE I.

The Agglutination of Strains of Hemolytic Streptococci from Erysipelas, Scarlet Fever, and Pyogenic Infections with Erysipelas Immune Sera.

Agglutinated strain.	Agglutinating sera.																																							
	Serum I.* Dilutions.†						Serum II. Dilutions.†						Serum III. Dilutions.†						Serum IV. Dilutions.†						Serum V. Dilutions.†						Serum VI. Dilutions.†									
E I	4	4	4	4	4	4	4	4	4	4	3	±	4	4	4	3	3	2	4	4	4	4	4	4	4	4	4	3	2	–	4	3	3	2	1	–				
E II	2	1	1	1	–	–	4	4	4	4	2	1	3	2	±	–	–	–	2	2	1	1	–	–	1	–	–	–	–	–	2	1	–	–	–	–				
E III	4	4	4	4	3	2	4	4	4	4	3	1	4	4	4	3	2	1	4	4	3	3	3	2	4	3	3	1	–	–	4	4	4	1	–	–				
E IV	4	4	4	3	2	2	4	4	4	4	3	–	4	4	3	2	–	–	4	4	4	4	4	3	4	4	4	3	1	–	4	4	3	–	–	–				
E V	4	4	4	3	1	±	4	4	4	3	2	–	4	4	3	2	1	–	4	4	3	3	1	–	4	4	4	4	4	3	4	4	4	4	4	3				
E VI	4	4	4	4	2	1	4	4	4	3	1	±	4	4	4	3	1	±	4	4	3	3	2	1	2	2	1	1	1	–	4	4	4	3	2	1				
E VII	4	4	4	4	2	1	4	4	4	4	2	1	4	4	3	2	1	–	4	4	2	2	1	–	2	2	1	1	1	–	3	2	1	–	–	–				
E VIII	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				
E IX	4	4	4	4	2	1	4	4	3	2	1	–	4	4	3	2	1	–	3	2	1	1	–	–	4	3	2	1	–	–	3	2	2	2	±	–				
E X	4	4	3	1	1	–	4	4	3	2	1	–	4	4	4	2	±	–	4	3	2	±	–	–	3	3	3	1	–	–	4	2	2	1	–	–				
E XI	4	4	4	2	1	–	4	4	4	3	2	1	4	4	4	3	1	–	4	4	4	4	3	2	3	3	2	1	–	–	3	3	3	2	1	–				
E XII	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				
E XIII	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				
S I	4	4	4	4	3	1	4	4	4	±	–	–	4	4	2	–	–	–	4	4	4	4	2	2	4	3	1	–	–	–	–	–	–	–	–	–				
S II	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				
S III	4	4	4	3	3	3	3	3	2	1	±	–	2	3	3	2	–	–	4	4	3	3	1	±	3	2	1	1	–	–	2	3	±	–	–	–				
S V	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				
S VI	3	3	2	1	1	1	–	–	–	–	–	–	3	3	3	3	2	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				
S VIII	4	4	3	2	2	1	4	4	2	1	1	1	4	3	3	3	1	1	3	3	2	1	1	1	4	3	2	1	1	1	2	2	1	±	±	±				
S IX	2	2	1	±	–	–	4	4	4	4	2	1	–	–	–	–	–	–	1	1	±	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				
S X	–	–	–	–	–	–	4	4	4	4	3	3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				
S XII	2	2	1	±	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3	4	4	3	2	2	2	2	1	±	–	–	2	3	2	2	1	±				
S XIII	2	1	–	–	–	–	4	4	4	4	3	1	–	–	–	–	–	–	2	1	±	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				

| | | |

strains. Several sera were prepared with each strain and those chosen which gave complete or nearly complete agglutination in dilutions of 1:1280, and incomplete reactions in higher dilutions. The tests of the titer were carried out with washed homologous antigens. Thirteen erysipelas strains, eleven scarlatinal strains, and ten strains from streptococcus infections were agglutinated with each of these sera. The reactions have been arranged in Table I, where the strains have been placed in groups and the source of each strain has been indicated by the initial letter, E, S, or P.

Of the group of thirteen erysipelas strains ten are agglutinated by all the erysipelas sera, occasionally to a greater degree than the homologous strain, but usually in a slightly lower titer. Certain strains apparently agglutinate poorly, yet are capable of stimulating the production of strong antibody. Three of the thirteen strains are not agglutinated by any of the sera. These results are quite similar to the results previously observed with scarlatinal strains and immune sera and indicate that, as in the case of the scarlatinal strains, erysipelas strains are closely related.

Of the eleven scarlatinal strains two agglutinate with none of the erysipelas sera, two with all, and the remaining seven with one or more sera but in an irregular fashion. None of the ten pyogenic strains agglutinate with all the erysipelas sera, and few with more than three. Four pyogenic strains are not agglutinated in any serum.

Of the 204 agglutination reactions in Table I, 78 are with erysipelas strains, 66 with scarlatinal strains, and 60 with pyogenic strains. Since 77, 48, and 21 per cent of the reactions in these respective groups are positive, we believe that the streptococci from erysipelas constitute a group of bacteria closely related antigenically, but that the group is not absolutely delineated from the scarlatinal or from the pyogenic strains. The group is obviously more related to the scarlatinal strains than to strains from common pyogenic infections.

The nature of the agglutinin occurring in these immune erysipelas sera was tested by a series of cross-absorption and agglutination reactions. The sera were absorbed with each of the thirteen strains, then homologous and several heterologous erysipelas strains were tested for agglutination by the absorbed sera. Two of the sera, E I and E II, were tested throughout the complete series of strains.

TABLE II.

Absorption Reactions with Erysipelas Serum I. Serum Absorbed by Each of Thirteen Erysipelas Strains and Tested against Seven Strains for the Presence of Agglutinin.

| Absorbing strain. | Agglutination of strains with Serum I absorbed by marginal strains. | | |

TABLE III.

Absorption Reactions with Erysipelas Serum II. Serum Absorbed by Each of Thirteen Erysipelas Strains and Tested against Seven Strains for the Presence of Agglutinin.

| Absorbing strain. | Agglutination of strains with Serum II absorbed by marginal strains. | | |

TABLE IV.

Agglutination and Absorption Reactions with Scarlatinal Serum III. Serum Absorbed by Scarlatinal Strains and Tested with Homologous and Heterologous Scarlatinal Strains for the Presence of Agglutinin.

Strain.	Agglutination of marginal strain in Serum S III. Dilutions.*						Agglutination of strains with Serum III absorbed by the marginal strain.						
							Agglutinated strains.	Dilutions.*					
S I	4	4	4	3	2	1	S I, II, IX	–	–	–	–	–	–
							S VI, XII	±	±	±	–	–	–
							S III	4	4	4	2	–	–
S II	2	1	–	–	–	–	S I, II, IX	–	–	–	–	–	–
							S XII	±	±	±	–	–	–
							S III	4	4	3	1	–	–
S III	4	4	4	4	4	3	S I to S XXII	–	–	–	–	–	–
S V	–	–	–	–	–	–	S I, II, IX	±	–	–	–	–	–
							S III	4	4	3	1	–	–
S VI	3	3	3	3	2	–	S I, II, IX	–	–	–	–	–	–
							S III	4	4	2	1	–	–
S VIII	4	4	4	4	4	3	S I, II, VIII	–	–	–	–	–	–
							S III	1	1	–	–	–	–
S IX	3	3	3	3	2	±	S I, VI, IX, XII, XIV	–	–	–	–	–	–
							S III	4	4	4	3	2	–
S X	–	–	–	–	–	–	SI, II, VI, IX, XII	–	–	–	–	–	–
							S III	4	3	1	–	–	–
S XI	2	2	1	–	–	–	S I, II, XI, XII, XIV	–	–	–	–	–	–
							S III	4	4	3	1	1	–
S XII	4	4	4	4	4	3	S I, IX, XI, XIV	–	–	–	–	–	–
							S III	3	4	3	1	–	–
S XIII	3	3	2	2	1	1	S XIII	–	–	–	–	–	–
							S III	4	4	4	3	2	–
S XIV	4	4	4	4	3	2	SI, VIII, IX, XII, XIV	–	–	–	–	–	–
							S III	4	4	3	3	–	–
S XV	4	4	4	4	3	1	S I, VIII, XII, XV	–	–	–	–	–	–
							S III	–	–	–	–	–	–
S XVI	–	–	–	–	–	–	S I	3	4	3	3	–	–
							S IX	1	2	2	2	1	–
							S VIII	3	4	3	2	–	–
							S XIV	4	4	4	3	1	–
							S III	3	3	2	1	–	–
S XVII	3	1	±	±	±	±	S I	±	–	–	–	–	–
							S III	4	4	4	3	1	±
S XVIII	–	–	–	–	–	–	S III	4	4	4	3	1	±
							S IX	±	–	–	–	–	–
							S VIII	4	4	4	3	2	–
							S XIV	3	3	4	3	3	–
S XIX	–	–	–	–	–	–	S I	–	–	–	–	–	–
							S III	4	4	3	3	1	–
S XX	2	2	±	±	±	±	S I	–	–	–	–	–	–
							S III	4	3	2	–	–	–
S XXII	2	3	3	3	2	1	S I	–	–	–	–	–	–
							S III	4	4	4	3	±	–

* The agglutination reactions are in a series of six dilutions, 1:40, 1:80, 1:160, 1:320, 1:640, and 1:1280. Controls in normal sera are negative and have been omitted.

The results of these absorption tests are found in Tables II and III. The agglutination of only seven strains of the series has been included.

We find that the agglutinin binding these strains together is, within the limits of the absorptive tests carried out, apparently common to

TABLE V.

Agglutination and Absorption Reactions with Scarlatinal and Erysipelas Sera and Scarlatinal, Erysipelas, and Pyogenic Strains.

Serum* absorbed.	Absorbing strain.	Strains agglutinated in absorbed sera.	Agglutinations. Dilutions.†					
E I	S VIII	E I	4	4	4	4	4	4
		E II, IV	1	1	−	−	−	−
		S I, III, VIII, XII	1	−	−	−	−	−
E I	E IV	E I	4	4	4	4	3	2
		E II, IV	±	−	−	−	−	−
		S I, III, VIII, XII	1	±	±	−	−	−
E I	P I	E IV	2	4	4	4	4	4
E I	P III	E IV	±	±	±	±	±	−
E II	S VIII	E II	4	4	4	4	3	1
		E I, IV	±	±	−	−	−	−
		S I, III, VIII, XII	±	±	−	−	−	−
E II	E I	E II	4	4	4	4	3	2
		E I, IV	−	−	−	−	−	−
		S I, III, VIII, XII	±	±	−	−	−	−
E II	P II	E IV	3	3	2	±	−	−
E II	P VIII	E IV	±	±	±	−	−	−
S III	S VIII	E I, II, IV,	±	±	−	−	−	−
		S I, VIII, XII	±	−	−	−	−	−
S III	E I	E I, II, IV	±	−	−	−	−	−
		S I, VIII, XII	±	±	±	±	−	−
		S III	4	4	4	3	2	−

* Homologous strains and sera are indicated by similar letters and numerals.

† The agglutination reactions are in a series of six dilutions, 1:40, 1:80, 1:160, 1:320, 1:640, and 1:1280. Controls in normal sera are negative and have been omitted.

all erysipelas strains. With one exception, Strain E VIII, which grew with a pellicle and was morphologically dissimilar to the remaining twelve strains, each strain regardless of its ability to agglutinate absorbed this common agglutinin for the heterologous strains. One

general exception is to be noted. Serum E I agglutinates the homologous strain after absorption with the heterologous strains, and Serum E II agglutinates both the homologous strain and Strain E XI after heterologous absorption. Since Strains E II and E XI give reciprocal absorption of agglutinin with Serum E II they are in all probability identical. Strain E I apparently has no exact counterpart among the thirteen strains. Strain XII absorbs Serum VI completely for Strain VI, but XII is not an agglutinable strain so that reciprocal reactions are not possible. The ultimate test to prove the identity of Strains XII and VI, and of Strains II and XI, would be reciprocal absorption of their respective sera, but the similarity has been assumed because only six sera of the proper titer were available. We conclude from these absorption tests that erysipelas strains have common antigenic properties but are not identical, although identical strains occur frequently in the group.

In Table IV we have tabulated the agglutination and absorption reactions of nineteen scarlatinal strains with scarlatinal Serum S III. 79 per cent of these strains agglutinate with this serum. Seventeen of the strains absorb the agglutinin for other heterologous strains while one (S XV), absorbs the agglutinin for the homologous strain, hence, is probably identical with it. Only one scarlatinal serum was available, but with this serum scarlatinal strains present relationships among themselves similar in every respect to the relationship existing among strains from erysipelas. The common agglutinin of the two groups has been studied in relation to the agglutination of pyogenic strains by erysipelas sera. Erysipelas and scarlatinal sera were absorbed with strains from each of the three groups studied, then tested for their agglutinative qualities with representative erysipelas and scarlet strains. We have found that pyogenic strains, scarlatinal or erysipelas strains, will absorb the heterologous agglutinin from immune scarlatinal or erysipelas sera if the sera agglutinate the absorbing strain. Difficulty was experienced in reading these reactions on account of a fine granulation in the lower dilutions of certain sera. We assume this was an artefact, but have indicated the instances in which it occurred by plus-minus signs since it may signify unabsorbed agglutinin.

DISCUSSION.

In discussing the results obtained in this series of reactions we must consider principally the bearing of the observations on the problem of streptococci in general, and attempt an explanation of the curious antigenic relationship of the groups studied to each other, and to other possible groups of streptococci. It appears that erysipelas strains agglutinate in a higher percentage of instances with erysipelas sera than do scarlet fever strains, and that the same sera agglutinate scarlet strains more frequently than pyogenic strains. Furthermore, while strains in the pyogenic group agglutinate irregularly with these sera, erysipelas strains give quite uniform results. If it were not for these variations in agglutination in the different groups, we should be inclined to believe that the scarlatinal and erysipelas strains together constituted a poorly defined group of streptococci with common antigenic properties.

Our experience with scarlatinal streptococci has been that the percentage of positive agglutinations obtained with scarlatinal serum, is approximately the percentage which we have obtained here with erysipelas sera and strains. The percentage obtained by crossing sera and strains of the two groups is lower. The absorption tests indicate that the agglutinin in all three instances is identical, but such an agglutinin is not consistent with the variations observed in the reactions obtained with the various sera. Furthermore absorption of agglutinin indicates antigenic similarity with but a relative degree of accuracy. The only possible explanation is to assume that the antigen in this instance is exceedingly complex and the nature of the complexity, whether of multiple antigens existing in certain combinations in certain groups of streptococci, or of innumerable chemical variations in a single complex antigen is at present a matter of pure conjecture. Likewise the antigenic nature of strains which appear identical, since they are capable of reciprocal absorption, is problematic. The similarity of these strains may depend upon specific substance similar to the specific substances in the pneumococcus groups, or may depend upon the occurrence of similar components in a complex antigen common to all of the strains. We are inclined to believe that similarity in a composite antigen is the more satisfactory

explanation. In many respects the scarlet and erysipelas groups of streptococci resemble closely related groups in the typhoid-colon group of Gram-negative bacteria. Probably hemolytic streptococci may be still further subdivided into similar closely related agglutinative groups having distinct relation to various clinical manifestations of streptococcus infection.

We have mentioned the necessity of assuming that the antigen in these streptococci is complex to explain the agglutination reactions described in the preceding paragraphs. Rather than assume innumerable variations in a single large antigenic molecule, we believe that an antigen made up of several loosely united components, offers the simplest and most satisfactory explanation. These components may be represented by letters, *a, b, c, d,* etc. If *a, b, c, d, e, f, g, h,* represent the elements in preponderance in scarlatinal strains, and *e, f, g, h, i, j, k, l,* those preponderating in the antigens of erysipelas strains, we can explain the close adherence of the strains within the groups and the crossing found with sera of one group and strains of the other. The chance agglutination of strains from wound infections, proven to be neither scarlatinal nor erysipelas strains would depend on the presence of some of the components most commonly present in scarlatinal or erysipelas strains. A single dominant component or a characteristic arrangement of a group of components in all probability gives specificity to the scarlatinal and erysipelas groups of streptococci. With further refinements in the technique of absorption we shall probably be able to rearrange these streptococci still further in subgroups and increase the specificity of agglutination. In the past agglutination has been one of the valuable methods of approaching clinical problems from the bacteriologic standpoint and we believe that in the study of streptococci and their relation to disease it still furnishes the most fruitful field in beginning a research of this character.

CONCLUSIONS.

1. Strains of hemolytic streptococci isolated from cases of erysipelas agglutinate in a high percentage of instances with erysipelas immune sera. When agglutination occurs with one serum, the strain usually agglutinates with all other erysipelas sera.

2. Erysipelas immune sera agglutinate strains from erysipelas in a higher percentage of instances than they agglutinate scarlatinal strains.

3. Strains from miscellaneous pyogenic infections may agglutinate in these sera, but the percentage of positive reactions is low and a strain usually agglutinates in but one or two of several sera.

4. Erysipelas strains which agglutinate in immune sera are not necessarily identical, although identical strains may occur. Similarly, identical strains may occur among scarlatinal strains agglutinated by scarlatinal immune sera.

5. Erysipelas strains form a closely related group of hemolytic streptococci. Scarlatinal strains form an equally compact group. The two groups are related antigenically but less closely related than the strains within the groups. These groups are related to pyogenic strains, but less closely than they are related to each other.

6. Erysipelas, scarlatinal, or pyogenic strains which agglutinate in erysipelas or scarlatinal sera are capable of absorbing the agglutinin for all other strains except the homologous strain and strains identical with it. Strains identical with the homologous strain absorb the agglutinin completely.

7. Erysipelas or scarlatinal strains may absorb the common group agglutinin from their respective sera, when they are incapable of agglutination in these sera.

8. The agglutinogen is probably of complex or composite nature, with characteristic variations in the scarlet fever and erysipelas groups.

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COMPENSATORY HYPERTROPHY OF THE ADRENAL CORTEX.*

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Experimental evidence concerning hypertrophy of the adrenal cortex and medulla following the removal of one gland is conflicting and uncertain. The results of previous workers have been summarized by Boycott and Kellaway (1), who, from a series of carefully controlled experiments, concluded that destruction of one adrenal in the rabbit is not followed by hypertrophy of either the cortex or the medulla of the other gland. Close inspection of their figures however leads one to think that these investigators were overconservative in the interpretation of their results and that in reality some hypertrophy did take place. It is true that there is a large per cent of variability in their figures but this appears to be due to the small number of animals used rather than to highly variable results. In the rat neither Harley (2) nor Poll (3) found compensatory hypertrophy. Wiesel (4) and Scott (5) both affirm the existence of compensatory hypertrophy of the cortical accessory adrenals following the removal of both glands in this animal. The data in each case were based upon doubtfully controlled experiments. Elliott and Tuckett (6) made careful weighings but only upon four rats and found no compensatory hypertrophy.

Methods.

Fifty albino female rats of exactly the same age were divided into a control group and an operated group, each of equal number. 14 days before these ani-

* This work was aided by the Wellington Gregg Fund.

mals became 90 days of age both groups were placed upon a special[1] diet which was maintained until the end of the experiment. The daily food intake, which was determined for cage units of five rats each, has been charted in Fig. 1. Each rat was weighed daily and the average weight curves of the two groups, which are shown in Fig. 1, are nearly alike.

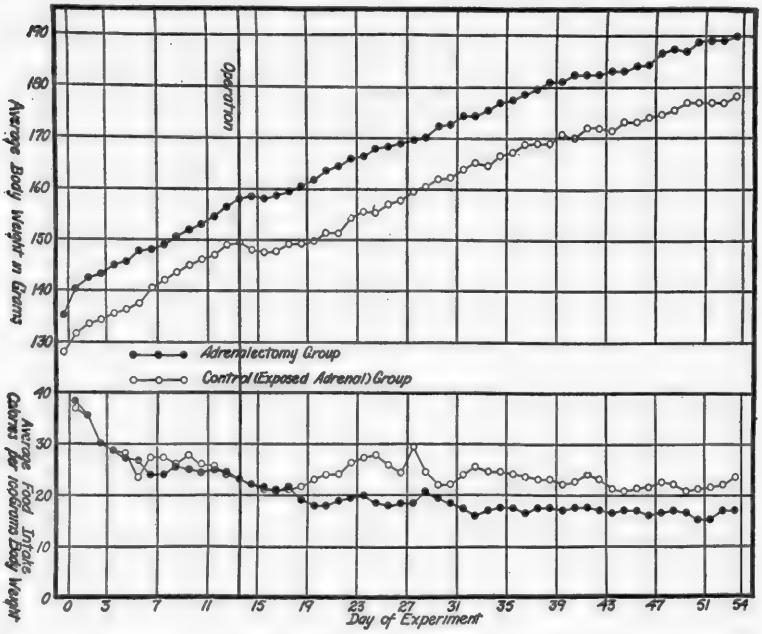

FIG. 1. Average daily body weight and average daily food intake.

[1] Adequate for growth and reproduction. The composition is as follows:

	per cent
Corn-starch	41.0
Casein	15.0
Lard	15.0
Cod liver oil	9.0
Wheat germ	10.0
Osborne and Mendel salt mixture	4.0
Dried yeast	4.0
Agar-agar	2.0

This contains 17.8 per cent protein, 24.9 per cent fat, and 42.2 per cent carbohydrate.

TABLE I.

No.	Control group.				Operated group.		
	Actual weight.		Weight of adrenal per 100 gm. body weight.		Actual weight.		Weight of adrenal per 100 gm. body weight.
	Left.	Right.	Left.	Right.	Removed.	Hypertrophied.	Hypertrophied.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	25	22	18.3	16.1	33–L.	40–R.	23.0
2	29	27	19.0	17.7	26–R.	29–L.	16.7
3	26	23	15.9	14.0	17–L.	52–R.	28.0
4	25	30	15.2	18.3	29–R.	41–L.	22.9
5	27	35	15.9	20.6	30–L.	39–R.	24.4
6	26	27	15.0	15.6	35–R.	50–L.	30.9
7	19	18	9.6	9.1	18–L.	41–R.	23.2
8	27	20	17.5	13.0	19–R.	38–L.	25.2
9	18	23	11.4	14.5	24–L.	37–R.	23.3
10	24	15	15.4	9.6	23–R.	48–L.	29.1
11	17	28	10.9	18.0	24–L.	36–R.	22.0
12	32	17	18.1	9.6	25–R.	32–L.	17.6
13	23	15	15.8	10.3	28–L.	32–R.	16.5
14	42	19	26.9	12.2	35–R.	44–L.	24.7
15	19	23	12.2	14.7	20–L.	45–R.	23.1
16	21	19	14.8	13.4	25–R.	46–L.	22.9
17	24	19	16.2	12.8	19–L.	43–R.	22.6
18	19	25	11.9	15.6	30–R.	43–L.	25.7
19	26	23	16.6	14.7	29–L.	40–R.	23.8
20	26	18	15.9	11.0	23–R.	38–L.	19.3
21	24	31	13.5	17.4	19–L.	42–R.	24.6
22	30	35	17.0	19.8	29–R.	33–L.	20.4
23	26	27	16.8	17.3	22–L.	39–R.	22.7
24	28	20	17.9	12.8	25–R.	31–L.	17.8
25	27	29	12.3	13.2	26–L.	36–R.	21.4
26	29	32	16.4	18.1	30–R.	39–L.	21.1
27					21–L.	35–R.	20.6
Average..	25.3	23.8	15.6	14.6	25.3	39.6	22.7
	24.6		15.1				

	Control group.	Operated group.
Standard deviation	5.44	5.48
Probable error of the average	0.51	0.71
Coefficient of variability	22.09	13.84

The stimulus to compensatory hypertrophy was given by the removal of one adrenal from each rat of the operated group. This was done 14 days after the start of the experiment; that is, when each rat was exactly 90 days old. At this age it has been shown (7) that the histological structure and the volume relations of the adrenal cortex and medulla have reached the adult condition. In the control group one adrenal was exposed. In both groups alternate glands were chosen on each successive rat for either exposure or removal under ether anesthesia. After removal each gland was freed of any adherent tissue or fat, with the aid of fine eye forceps, and the glands removed were weighed immediately on a torsion[2] balance to within 0.5 mg. They were then fixed in 10 per cent neutral formalin.

40 days was arbitrarily chosen as the period of time in which to determine the ability of the adrenal gland to undergo compensatory hypertrophy. At the end of this period all of the animals of both groups were killed with ether, quickly opened, and the adrenals removed and carefully weighed as before. In the case of the rat adrenal volume changes have been found comparable (8) to results expressed by weight.

FIG. 2. Gross appearance of adrenals selected at random from each group. (*a*) Normal adrenals from control group. (*b*) Hypertrophied adrenals from operated group.

RESULTS.

The results are given in Table I. These give indisputable evidence of adrenal hypertrophy following unilateral adrenalectomy, either of the cortex, medulla, or the whole gland. This is shown visually in Fig. 2. The actual difference between the averages of the adrenal weights of the control and operated groups is 15.0 mg., while the probable difference of the averages determined by the usual method is only 0.88 mg. The degree of compensatory hypertrophy, that is the difference between the average value of the single control adrenals and the average value of the hypertrophied adrenals, is expressed as the percentage of the average value of the single control adrenals. In this experiment, 40 days after the removal of one adrenal, the

[2] Hartmann-Braun torsion balance of 500 mg. capacity.

compensatory hypertrophy of the other gland amounted to 61 per cent. Since the average body weights of the control and operated groups were practically alike (Fig. 1) the results in Table I determined from actual weights and adrenal weight in relation to body weight are essentially the same. Actual weights only are therefore considered here.

A comparison of the average value of the adrenal weights of the control group with that of the adrenals removed from the operated group at 90 days of age makes it evident that little if any growth of the glands of the control group took place during the course of the experiment. From Donaldson's table (9) an average adrenal weight increase of 8 mg. might be expected during this 40 day period. Even though this growth had taken place a large degree of hypertrophy

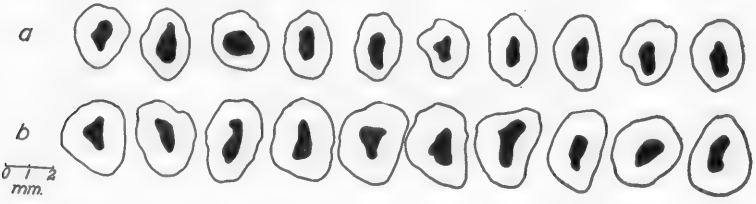

FIG. 3. Camera lucida outlines of adrenal medulla and cortex. (*a*) Normal adrenals of control group. (*b*) Hypertrophied adrenals of operated group.

would still exist. In order to obviate the necessity of considering the right and left glands separately alternate glands were used for exposure in the control group and removal in the operated group. This was not absolutely necessary for no difference has been found between the right and left adrenals of the control group. The probable difference of the average weights of the two sides is 0.97 mg. and the actual difference is only 1.50 mg.

The only compensatory adrenal hypertrophy which has been found in the rat, that of accessory adrenals following total adrenalectomy (4, 5), consisted of an increase in cortical tissue. In our experiment likewise the hypertrophy was limited to the cortex. Ten unselected glands from each group were available for histological examination. Fig. 3 shows camera lucida outlines of the medulla and cortex of central sections of all twenty glands as determined by serial section.

The area of the whole section and of the medulla was determined in each case and then, treating these as center sections of spheres, the average volume of cortex and of medulla was determined for the ten glands of each group. The results form Table II. The relative volume of cortex and medulla, that is cortex 93 per cent and medulla 7 per cent, as determined from the average figures for the control group agree very well with similar figures of other observers determined by accurate reconstruction methods. In adrenals of female rats of the same age Jackson (7) found that the cortex comprised

TABLE II.

Cortex and Medulla Volumes in C. Mm. of Ten Adrenals from Each Group Calculated from the Area of the Central Section of Each Gland Treated as the Midplane of a Sphere.

	Control group.			Hypertrophied group.		
	Total gland.	Medulla.	Cortex.	Total gland.	Medulla.	Cortex.
Average	5.99	0.42	5.58	11.15	0.46	10.69
Standard deviation	0.74	0.11	0.69	1.37	0.10	1.32
Probable error of average	0.16	0.02	0.15	0.29	0.02	0.28
Coefficient of variability	12.50	26.20	12.36	12.29	21.73	12.34

	Whole gland.	Medulla.	Cortex.
Actual difference of the averages	5.16	0.04	5.11
Probable difference of the averages	0.33	0.03	0.32
Per cent compensatory hypertrophy	86.0	0.0	91.0

about 93 per cent and the medulla 7 per cent of the total gland volume. Another investigator (8) found 93.5 and 6.5 per cent respectively.

It is evident from Table II that the hypertrophy is limited to the cortex as there is no appreciable difference in the medulla volumes of the control and hypertrophied glands. When considered as hypertrophy of the entire gland the increase in tissue as we have already shown amounted to 61 per cent. This is due to a 91 per cent hypertrophy of the cortex. The irregular form of the adrenals lends a large degree of inaccuracy to this method of determining the region of hypertrophy. However, further evidence of the limitation of hypertrophy to the cortex is offered in cell measurements to follow.

It is conceivable that the increase in adrenal cortex is a result not of hypertrophy but of stimulated growth. In this case an increase in the number and not in the size of cells would be expected. Measurements of cell areas have been made in sections of normal and hypertrophied glands. Areas of every twentieth 6 micron section from ten adrenals of each group were counted. The number of nuclei in two areas 0.01 mm. square were determined in three parts of each section. The cells comprising the various cortical layers differ greatly in size. The zona glomerulosa is very narrow and no measurements were made of these cells. The cells of the zona fasciculata

TABLE III.

Average Number of Nuclei in 0.01 Sq. Mm. of a 6 Micron Section Determined from Measurements of Every Twentieth Section of Ten Glands from Each Group.

Area of gland	Control group.			Hypertrophied group.		
	Cortex—zona fasciculata.	Cortex—zona reticularis.	Medulla.	Cortex—zona fasciculata.	Cortex—zona reticularis.	Medulla.
No. of areas counted	75	52	39	108	66	41
Average number of cells	124	330	144	63	202	145
Standard deviation	26.44	50.89	30.59	20.03	75.02	31.85
Probable error of the average	2.06	4.76	3.30	1.30	6.23	3.36
Coefficient of variability	21.32	15.42	21.23	31.80	37.13	21.97

	Cortex—zona fasciculata.	Cortex—zona reticularis.	Medulla.
Actual difference of the averages	61.00	128.00	1.00
Probable " " " "	2.44	7.84	4.71

are normally larger and more variable in size than those of the zona reticularis adjoining the medulla and are considered separately. Two areas each were taken of the middle cortical zone, of the cortex adjoining the medulla, and of the medulla cells. The results in Table III show a definite increase in the size of the cortical cells, or at least a decrease in the number of nuclei per unit volume, and evidence of cortical hypertrophy which is not accelerated growth.

It is of great interest that no increase in the size of the medulla or its cells was found. No hypotheses are offered here relative to the failure of the medulla to hypertrophy as we propose carrying out further experiments in an endeavor to explain this finding.

SUMMARY.

1. Removal of one adrenal in the albino rat is followed by hypertrophy of the remaining gland. In the case of female rats 90 days of age this compensatory hypertrophy amounted to 61 per cent.

2. The increase in size of the whole gland is due entirely to hypertrophy of the cortex. This increase in cortical tissue amounted to approximately 91 per cent and was due in large part if not entirely to an increase in the size of the cells.

3. No increase in the size of the medulla or its cells was found.

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STUDIES ON THE HISTOGENESIS OF AUTOPLASTIC THYMUS TRANSPLANTATIONS.

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Plates 7 and 8.

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There are many mooted problems connected with the thymus gland in spite of the numerous studies dealing with the embryology, comparative anatomy, histology, and pathology of this organ. Kölliker (1) in 1879 drew attention to the fact that the thymus has an epithelial anlage from the branchial clefts, and since that time many attempts have been made to explain how from this epithelial germ had arisen the finished structure of the organ, with its important elements—the reticulum, Hassall's corpuscles, and free lymphoid-like cells. The lack of knowledge and the disagreement as to the origin and biological significance of the small thymic cell have been one of the main stumbling blocks towards a proper understanding of the functions of this organ. These cells have been the subject of contention among those who have worked in the field. Two schools have developed, each of which has during the last 25 years vigorously upheld its views. The Maximow-Hammar (2, 3) group supported by Rudberg (4), Jonson (5), Pinner (6), Pappenheim (7), and Danchakoff (8) believe in the lymphocytic nature of these cells, while the Maurer-Stöhr (9, 10) school claims that these cells are epithelial in origin, though they appear like mesodermal lymphocytic cells. In addition they believe that these cells are capable of reverting to their original epithelial state.

Since up to the present time no definite solution of the problem has been established, we believed that the method of autoplastic thymus transplantation with a detailed study of the histological changes which occur in the transplants from a few hours after their insertion to their

complete regeneration would throw some light on the origin of these cells.

From a review of the literature it is quite apparent that some authors utilizing autoplastic transplantation have successfully transplanted this gland subcutaneously, intramuscularly, subperitoneally, intraperitoneally, and into the spleen. However, none of the previous workers have studied in detail the cytological changes which occur in the regenerating transplants. In addition, many misconceptions and failures have developed because some authors have not taken cognizance of the biological differences between auto-, homo-, and heterotransplantation.

A Summary of the Literature.

Dudgeon and Russell (11) performed thymus homotransplantations on the peritoneum, introducing large pieces of the gland, but these were quickly absorbed. They used one rabbit, one kitten, four puppies. Hart and Nordmann (12) also did homotransplantations on dogs, and placed the thymus in the subcutaneous tissues of the abdominal wall. Their transplants were also absorbed. Gebele (13) carried out homo- and heterotransplantations of the thymus. His homotransplantations on dogs led him to the conclusion that the thymus is synergistic with the thyroid, but the transplants were not examined histologically. In his heterotransplantation work Gebele introduced into the abdominal walls of three dogs large pieces of thymus from three cases of Graves' disease. Examination of these transplants 5 to 6 weeks later showed complete resorption of the thymus tissue. Bircher (14), on the other hand, claims to have produced Basedow's disease in five dogs following implantation of large pieces of thymus, from cases of status thymicolymphaticus, cretinism, and neurasthenia.

Tongu's (15) autoplastic transplantations were carried out on 20 dogs and 50 rabbits, but many of his animals died soon after operation. The transplants were inserted usually on the peritoneum at the root of the mesentery. In only three dogs and eight rabbits did his transplants take. Renton (16), on the other hand, studied both auto- and homotransplants of the thymuses in very young rabbits and guinea pigs. He reported that all his autoplastic transplants took easily and that two homotransplants out of six were actively growing after 53 days. Grimani (17) also did autoplastic transplantations on rabbits. His work was concerned chiefly with the influence of thymic transplantation on the survival of the animal and on changes in the blood and bone. There were no histological studies of the transplants. Scalone (18) reports failures even with autotransplantations, which were absorbed within 20 days. Marine and Manley (19) autotransplanted the thymus of eight rabbits and studied the relation of transplantation to sexual maturity. They had four actively growing transplants at

the end of 4 months, one being present and active at 735 days. But in their work no special attention was directed toward histological studies.

Yamanoi's (20) recent paper reports probably the most detailed studies to date on autoplastic thymus transplantation. He transplanted the thymus into the spleen, and of sixteen animals which lived more than 14 days, eight had growing transplants. The transplants of the animals which died during the first 2 days were described and the degenerative changes noted. He also studied transplants 14, 21, 28, 32, 116, 117, and 163 days after their insertion. It is evident that while Yamanoi's experiments show survival of the transplants for long periods of time, he could not trace in detail the regenerative processes since, as we will point out, they take place in the main between the 4th and 9th days after transplantation.

Methods.

In this work young tame albino rats were used, varying in age from 38 days to 45 days. These rats were reared in the laboratory. All operations were performed under ether anesthesia. The operative field was shaved and this was followed by wiping the field with sponges dipped in bichloride solution. For operative removal of the thymus, the sternum was split down its middle to about the third rib, the bone was retracted, and the triangularis sterni muscle separated. The gland was exposed and separated with as little trauma as possible from the pleuræ and mediastinal fat, and it was immediately placed in sterile physiological salt solution which was kept at temperatures between 37° and 39°C. The abdominal wall was then prepared for the transplantation, and fragments of the gland measuring about 3 mm. in diameter were inserted into prepared pockets in the abdominal muscle. Great care was exercised to avoid hemorrhage into these pockets. The site of the transplant was indicated by closing the mouth of the pocket with a silk ligature. Four transplants were usually inserted into each animal.

In removing the transplants, the animal was anesthetized with ether, after which it was killed and a complete autopsy was done. Three transplants were fixed in Helly's, Bouin's, and strong Flemming's solutions, while the fourth was fixed either in 10 per cent formalin or 95 per cent alcohol.

The tissues were embedded in paraffin and cut serially. The sections were stained with hematoxylin-eosin, Heidenhain's iron-hematoxylin, Flemming's triple, and Van Gieson's stains. The frozen sections were stained with Sudan III. For general purposes we found fixation with Bouin's and staining with iron-hematoxylin highly satisfactory, but the clearest histological pictures were ob tained after Flemming's fixation.

Presentation of Data.

This study is based on an examination of 212 transplants removed from 53 young rats and fixed and stained by the methods enumerated

above. The transplants were examined at approximately 12 hour intervals until the 106th hour, and from then on at 24 hour intervals until the 16th day. Descriptions of the successive stages of degeneration, regeneration, and growth of the transplants follow.

12½ Hours.—(Fig. 1.) There is irregular clumping of the small thymic cells with pycnosis and caryorrhexis of many of their nuclei. The nuclear debris is collected in small masses throughout the transplant. The small round thymic cells at the periphery of the transplant are still fairly well preserved. The reticular cells do not show a corresponding disintegration, these cells retaining, for the most part, their normal histological appearance. The vessels at the periphery of the transplant are dilated and engorged and contain a large number of leucocytes. An occasional polymorphonuclear leucocyte finds its way into the transplant. There is as yet no evidence of hemorrhage into the transplant or new blood vessel formation.

27 Hours.—There is massive agglutination of the small thymic cells except at the periphery of the transplant. The zone of preserved cells at the periphery is irregular, but these cells seem to be surviving and are probably being nourished by the lymph. Caryorrhexis of the nuclei is more pronounced than in the preceding stage. The reticular epithelium is prominent, and many of the cells show frank evidence of beginning destruction. Numerous capillaries have appeared particularly in the centers of the transplants. The peripheral vessels about the transplant are still dilated and engorged. Numerous enlarged lymphatic vessels are seen filled with thymic round cells which have emigrated from the transplant. Large numbers of round cells also are seen between the muscle fibers and these cells are undoubtedly wandering out from the transplant. The arterioles show definite adventitial thickening. There is edema and fatty infiltration of the surrounding muscle fibers. Numerous polymorphonuclear leucocytes have infiltrated the transplant and surround the larger masses of agglutinated nuclear material. The leucocytes are actively phagocytic and many are seen filled with nuclear debris in the vessels about the transplant.

36 Hours.—(Fig. 2.) There is further progression in the destruction of both the small round thymic and reticular cells, the most pronounced disintegration being evident in the center of the transplant. The infiltration of polymorphonuclear leucocytes into and about the transplant has increased, and the leucocytes are actively phagocytic. At the periphery of the transplant some apparently surviving small thymic cells are still seen.

48 to 51 Hours.—There is at this time almost complete disappearance of the small thymic cells except for some cells at the periphery. The transplant areas are filled with polymorphonuclear leucocytes. The reticular cells are beginning to proliferate and are increasing very rapidly by mitotic division particularly at the periphery of the transplant. These proliferated reticular cells are spindle-shaped, or appear like epithelioid cells. In some places the reticular epithelial

cells are arranged in whorls. Newly formed blood vessels extend into and surround the transplant. The engorged lymphatic vessels described in the earlier hours are not seen, and only very few small round cells are now seen between the muscle fibers.

60 Hours.—Sections show progression from above stage with almost complete absence of the small thymic cells, and marked reticular proliferation. Epithelial elements in strand-like formation or whorls are replacing the original transplant mass. While these cells were spindle-shaped in the previous stage they are now becoming polyhedral. Small round cells appear in the more branched reticular network. These small round cells have no apparent relation to blood and lymphatic vessels, and the blood vessels do not contain an excess of nucleated cells. These facts argue against the infiltration of small round cells into the reticulum from the blood and lymphatic channels. There is a very active mitosis of the reticular epithelial cells and the mitotic figures are most frequent where the small lymphoid-like cells appear first in the stroma.

75 Hours.—(Fig. 3.) The transplant is made up almost completely of reticular cells in whorl formation. There is active mitosis in these cells.

106 Hours.—(Fig. 4.) At this stage the reticular cells show further enlargement with active mitosis, the transplant being formed almost exclusively by these cells. Small round cells appear between the reticular cells as in the 75 hour transplants, but none of these cells are present in excess in the blood vessels or lymphatics. There is as yet no mitotic division of the small thymic cells. Langerhans giant-like cells are seen about the transplant due to disintegration of muscle fibers. There is marked thickening of the adventitial and muscular coats of the blood vessels about the transplants. A few leucocytes are still seen in the transplant and the surrounding muscle.

5 Days.—(Fig. 5.) A great number of small thymic cells appear in a hyperplastic and very vascular reticulum. Some of the transplants still show in areas solid pearl-like formations. In some of the sections a few multinuclear Hassall's corpuscles are seen.

6 Days.—The first evidence of thymic lobules appears, and these lobules are small, being made up mostly of round thymic cells. There is yet no differentiation into cortex and medulla.

7 Days.—(Fig. 6.) There is further regeneration and the small thymic cells are closely packed and mask the reticulum. There are some well developed Hassall's corpuscles in the transplant. At this stage the first evidence of differentiation of the regenerated lobules into the cortex and medulla becomes apparent.

8 and 9 Days.—There is still further differentiation of the regenerated lobules with active mitosis of the reticular cells.

10 Days.—The transplants show completely regenerated thymic lobules. The reticular cells are almost completely masked due to overcrowding by the small thymic cells, and these show mitotic division figures. In several of the transplants some well formed Hassall's corpuscles are seen.

11 and 12 Days.—(Fig. 7.) The sections show regenerated transplant areas with distinct lobules and medullary centers. As in the above stage the small thymic cells are very abundant and obscure the underlying reticulum. There is active mitosis throughout the transplant.

13 and 14 Days.—In addition to the regenerative changes described above, one transplant shows several duct-like spaces lined by low cuboidal epithelium containing some debris. These structures are undoubtedly regenerated ducts which one occasionally sees in the normal rat thymus.

15 and 16 Days.—(Fig. 8.) Well developed, large transplant areas showing closely aggregated small thymic cells. There is division of the lobules into cortical and medullary zones.

DISCUSSION.

Our work shows that autoplastic transplants of the thymus gland undergo a series of changes characterized by degenerative phenomena which begin within a few hours and reach their height in about 2 days. These destructive changes affect the small thymus cells first, and then the reticular cells. At the end of the first 24 hours the transplant becomes vascularized and phagocytosis of the debris by polymorphonuclear leucocytes begins. Regenerative changes begin after 48 hours and these are characterized by proliferation of the reticular cells, which as regeneration progresses, leads to almost complete replacement of the transplant area by these cells so that the transplant takes on an epithelial appearance. During the 3rd day the small thymic cells first appear between the proliferated reticular epithelium cells, apparently as a result of enlargement and division of these latter cells. At about the 6th day thymic lobules are formed, and during the 7th day there is beginning differentiation of these lobules into cortex and medulla. At the end of 2 weeks all active transplants show thymic lobules made up of cortex and medulla, which differ from the normal thymic lobules of young animals only in that they are smaller and irregular.

The same series of changes have been observed independently by Rudberg and Jonson in their studies on the regenerative changes in the thymus gland following acute and chronic inanition and x-ray injury. In spite of the fact that we have observed on the whole the same regenerative changes as these authors, our views as to the origin of the small thymic cell are at variance with theirs. Both Jonson and Rudberg support Hammar in the belief that the small thymic cells

are lymphocytes and that these cells make their appearance in the gland during regenerative phenomena, by the process of infiltration. A study of their work shows, however, that these authors have no convincing or direct evidence for their conclusions.

Jonson in 1909 studied the effect of acute hunger and chronic inanition on the involution of the thymus in young rabbits whose ages varied from 6 weeks to 5 months. He found, as have others, that chronic hunger rapidly involutes the thymus. He states that the disappearance of the cortical cells during hunger is dependent upon the emigration of these cells from the organ through the lymph and venous channels of the medulla. As a result of this, the thymus loses its lymphocytic appearance, becoming epithelial in character. When the animal is given a full diet there is rapid regeneration of the gland. In the earliest stages of regeneration there is no differentiation of the thymus into cortex and medulla, and the parenchyma has a cortical appearance. Jonson further states that the regeneration is dependent upon the immigration of lymphocytes from lymph and blood channels into the epithelial network. When regeneration has advanced further, mitotic division of these lymphocytes occurs. Only after the 16th day of normal nutrition is there any evidence of medullary formation. The medulla is then formed by the hypertrophy of the reticular cells *in situ*, and during the 3rd week the medulla appears normal.

Assuming that Jonson's observations of the emigration of the lymphocytes through the blood and lymphatic channels of the medulla are correct, still there is no reason why these cells should return to the thymus through these same channels. Jonson has also not been able to demonstrate an excess of lymphocytes in the blood and lymphatic channels during regeneration. His conclusions are based only upon the evidence that the small thymic cells are seen in abundance in the vicinity of the blood vessels and perivascular lymphatics during regeneration.

That the reticular cells are more resistant than the lymphocytes is shown by the fact that after prolonged inanition the gland appears epithelial in nature, and that at this time, the mitotic figures remaining in the gland occur almost exclusively in the reticular cells. If, as Jonson describes, mitotic division figures appear in the lymphocytes only after many days of forced feeding following inanition, then to explain the infiltration of the lymphocytes into the reticulum, one must bring in some other influence, as for instance, chemotaxis if we do not accept their origin from the reticular cells. That they arise

from the reticular cells is further supported by the fact that these cells undergo rapid mitotic division during regeneration. Then as regeneration progresses, instead of the reticular cells becoming more prominent, the organ becomes more and more lymphocytic in nature.

Our own observations confirm this point. After 48 hours there is marked reticular proliferation and mitotic division of the reticular cells, but from then on round cells appear between the reticular cells and by the 6th day lobules composed almost entirely of round cells appear. Even at this stage it is unusual to find mitotic figures in the round cells.

Rudberg comes to the same conclusions as Jonson, but he studied regeneration of the thymus following x-ray injury. He worked with young rabbits 1 to 4 months of age, and concluded that in the early stages of regeneration lymphocytes appear in the perivascular connective tissue and that this connective tissue is related to the lymph channels. These lymphocytes crowd into the centers of the thymic lobules and spread toward the periphery. Later, mitotic division figures appear in the lymphocytes. After the increase of lymphocytes and reticular cells has progressed sufficiently the division of the lobules into cortical and medullary zones takes place. Rudberg states that the small thymic cells could not arise from the reticular cells because the reticular cells are degenerating when the lymphocytes first appear. It should, however, be pointed out that while the reticular cells show degeneration when the lymphocytes first appear, the degeneration at any given time involves only groups of the reticular cells. Then again, the same general criticisms apply to Rudberg's work as to Jonson's.

The other studies dealing with the origin of the small thymic cells are essentially embryological and hematological. On the basis of numerous studies on human thymuses and those of fishes, Hammar came to the conclusion that the thymus round cell or thymic lymphocyte is a blood lymphocyte. His results are based on the facts that histologically both cells have basophilic protoplasm, both are ameboid, and both react alike to x-ray. Hammar also traced the early invasion of the thymus anlage by lymphocytes.

In spite of these apparently intensive studies, Stöhr has denied the correctness of Hammar's conclusions. In addition, the literature contains several other papers dealing with the embryology of the thymus in which the authors try to show that the thymus round cell is not a lymphocyte (21, 22, 23). Stöhr claims that his work on amphibians, humans, mice, cattle, sheep, pig, and the cat confirms the belief of the local epithelial origin of the small thymic cell. He is convinced that they are always epithelial and remain epithelial, although they may look like lymphocytes.

The recent investigations of Sabin (24) and those of Lewis and Lewis (25) on the white cells of the blood would seem to throw additional light on this problem. These authors studied the changes in the leucocytes of the blood in hanging drops

and in incubated blood, and found that the leucocytes become transformed into macrophages, epithelioid cells, and giant cells. They also observed the transformation and growth of the leucocytes into macrophages, epithelioid cells, and giant cells in the blood of the chick embryo, young chicken, adult hen, mouse, guinea pig, dog, and human. In all bloods examined the leucocyte developed first into a large phagocytic cell, then into a primitive mesenchyma cell, and still later into an epithelioid cell. Also some of the leucocytes became giant cells of the Langerhans type, while others appeared like the clasmatocytes of the connective tissue.

Clark and Clark (26) in 1920–23 on the basis of quite similar studies came to the conclusion that the polymorphonuclear cells resemble fibroblasts at sites of inflammation.

The investigations of these workers show definitely that the macrophages, epithelioid cells, and giant cells do arise from the leucocytes of the blood without, in this case, any possibility of participation in the phenomenon by the endothelium or the connective tissue.

These studies show that biologically the blood cells are closely related to a number of cells which are morphologically quite different. They would further indicate that while the small thymic cell resembles a lymphocyte, morphological similarity necessarily does not argue in favor of a biological relationship. Our experiments offer further substantiation of this view. Careful histological studies show no invasion of lymphocytes. Furthermore, the time of appearance of the small thymic cells coincides with the period of most active division of the reticular cells.

The studies on spleen transplants by Marine and Manley (27) which showed complete regeneration, with all the characteristic anatomical features of fully developed spleen, took place from extremely small fragments. This work would indicate, as these authors pointed out, that although anatomically the spleen is a highly complex structure, biologically all the major elements of this organ are simple, as indicated by its uniform and marked regenerative capacity.

CONCLUSIONS.

1. The thymus gland of young rats autotransplants easily when inserted in the muscles of the abdominal wall.

2. Destructive changes take place during the first 48 hours, when regenerative changes set in.

3. The regeneration is usually complete by the 14th day, when the new formed thymic lobules show differentiation into cortical and medullary zones.

4. The small thymic cells arise from the reticular epithelium in the regeneration of the transplant.

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EXPLANATION OF PLATES.

PLATE 7.

FIG. 1. 12 hours. Irregular clumping of small thymic cells with pycnosis and caryorrhexis of many of their nuclei.

FIG. 2. 36 hours. Shows massive agglutination of nuclear material in the center of a lobule due to destruction of both the small round thymic and reticular cells.

FIG. 3. 75 hours. Reticular cell regeneration has progressed to a considerable degree. A few leucocytes are still scattered throughout the figure. There is new blood vessel formation.

FIG. 4. 106 hours. Further enlargement and prominence of the reticular cells. Small round cells appear between the reticular cells.

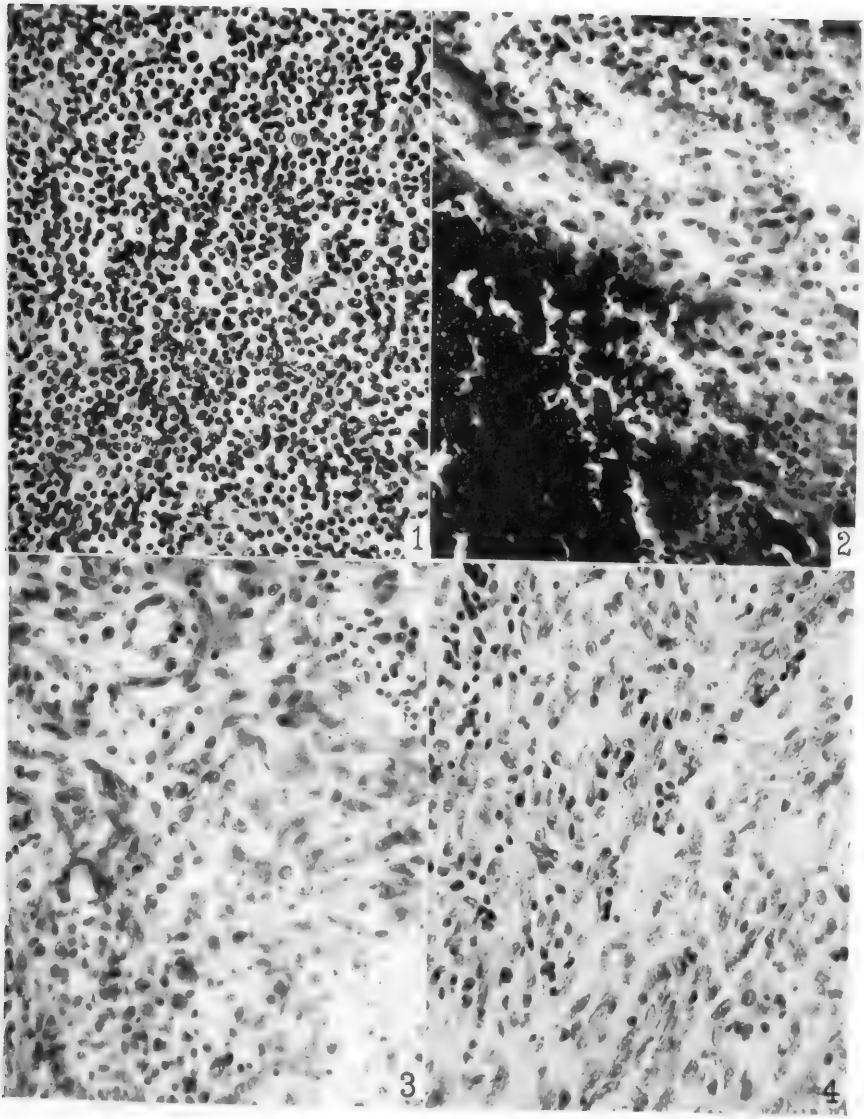
PLATE 8.

FIG. 5. 5 days. The small round cells are becoming more abundant.

FIG. 6. 7 days. Further regeneration; the small thymic cells are closely packed and mask the reticulum.

FIG. 7. 12 days. A regenerated thymic lobule with cortex and medulla.

FIG. 8. 15 and 16 days. A well developed large transplant area showing numerous lobules with cortical and medullary zones.



(Gottesman and Jaffe: Autoplastic thymus transplantations.)

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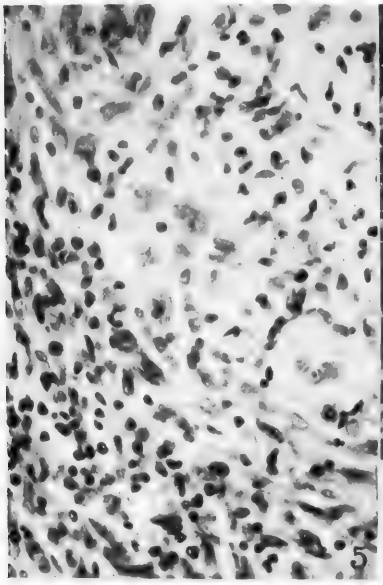

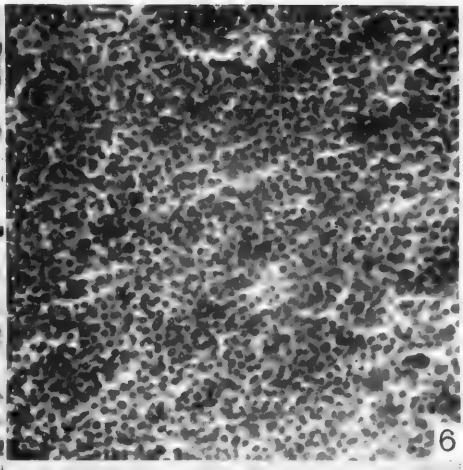

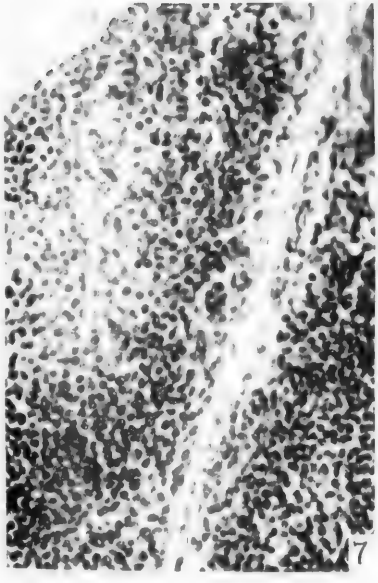

(Gottesman and Jaffe: Autoplastic thymus transplantations.)

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STUDIES IN THE COMMON COLD.*

I. Observations of the Normal Bacterial Flora of Nose and Throat with Variations Occurring during Colds.

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INTRODUCTION.

Although the problem of the common cold is now fully recognized as most important, no completely acceptable explanation of its causation has been presented. If we exclude the group of cold-like disturbances of allergic origin, the consensus of opinion is that the common cold is infectious in nature; but the work of many investigators has as yet failed to assign the etiological rôle to any one microorganism.

From time to time a causative relationship has been ascribed to a variety of bacteria. Pfeiffer (1), Voorhees (2), Tunnicliff (3), Cecil (4), Cautley (5), Mackey (6), Floyd (7), and others have attempted to implicate one or more of the following organisms: *Micrococcus catarrhalis*, diphtheroids, streptococci, staphylococci, *B. pfeifferi*, pneumococci, and various anaerobes. The work of these investigators has been open to criticism largely because of their failure to include adequate control studies of the normal flora of the nose and throat. Kruse (8), and later Foster (9), reported the successful transmission of colds by the inoculation of healthy individuals with nasal washings, obtained from colds, which had been passed through Berkefeld filters; the latter described the cultivation of minute "globoid" organisms to which he assigned the causative rôle. Schmidt (10), Williams *et al.* (11), Branham and Hall (12), and Robertson and Groves (13) failed to confirm these results. More recently Olitsky and McCartney (14) have again carried out similar successful transmission experiments; they failed to obtain the "globoid

* This study was made possible by a grant from the Chemical Foundation, Inc.

bodies" described by Foster, but did recover three types of filter-passing Gram-negative bacilli to which, however, they assigned no etiological rôle in view of their incidence in normals (15).

In searching for the causative agent in respiratory disease, the problem is seriously complicated by the fact that the nose and throat normally harbor a variety of organisms, some more or less constantly present and others transient in nature. In order, therefore, to interpret correctly the importance of bacteria present in respiratory disease, it is imperative that the investigator be conversant with the normal flora in health.

To this end, Williams *et al.* (11), in their intensive study of respiratory disease, made a number of cultures from normals and compared these results with the findings in disease. Bloomfield (16) went a step further. He made a preliminary survey of the flora of a group of normals over periods of 1 to 3 months, and then studied the flora in a series of colds and compared the results in the two groups. Jordan (17) made a similar study over a period of 2 to 3 weeks.

In the present preliminary study we are reporting observations of the flora in a group of normals over a comparatively long period, and, with this background for comparison, determinations of the quantitative and qualitative changes, if any, that took place in the course of the colds occurring in the group. We have been particularly interested in (1) what constitutes the normal flora of the nose and throat, (2) the incidence of potential pathogens and their significance, and (3) the variations from normal in the course of colds.

Methods.

Material.—Thirteen individuals were studied between October, 1924, and June, 1925. Of these, six were followed approximately 9 months, and seven from 4 to 5 months. Five were physicians, three working in the bacteriological laboratories and two in the chemical, and all in frequent contact with patients suffering from infectious diseases of all sorts; two were laboratory assistants, one bacteriological and one chemical; six were nurses on active ward duty.

Frequency of Cultures.—Cultures were made weekly when the individuals were in normal health, and daily when colds occurred; in addition, a few daily cultures were taken when sore throats or tonsillitis made their appearance.

Sources of Culture Material.—Nasal cultures were obtained by passing slightly curved sterile cotton swabs (on wire) well back into the nostril, using great care to avoid contact with the external nares. Throat cultures were made by passing curved swabs over the posterior pharyngeal wall, up behind the soft palate, and over the fauces and tonsils.

Cultural Methods.—Both aerobic and anaerobic cultivation were carried out in most instances during the first half of the work; later, when the group was enlarged,

anaerobic cultures were done once a month. Anaerobiosis was obtained with the Brown modification (18) of the McIntosh and Fildes jar (19), the indicator being methylene blue in 1 per cent dextrose broth.

The culture material, obtained as noted above, was immediately rubbed on fresh 5 per cent rabbit blood agar plates; the same swab was used for both aerobic and anaerobic plating. Streaking was done with sterile bent glass rods. This method gave satisfactory discrete colonies and it was not found necessary to resort to any system of dilution.

Aerobic plates were examined after 20 to 24 hours and were studied again after 48 hours. Anaerobic plates were examined after 5 to 7 days. Each type of colony was fished and the organisms identified. This was done by plate inspection, morphological study, and complete bacteriological study whenever indicated. At the time of examination, the predominating and the second most common organisms were determined.

Classification of Bacteria.—Gram-negative cocci were taken as a group, strain differentiation not being attempted. Streptococci were classified as hemolytic and non-hemolytic. Staphylococci, both hemolytic and non-hemolytic, were classified according to their pigment formation. A large Gram-positive staphylococcus-like organism which grew in large, dull greyish colonies was designated as "large Gram-positive coccus" (16). Pneumococci were occasionally noted. This organism was searched for carefully and the low incidence may be attributed to the fact that mouse inoculation was not practiced. All strains of diphtheroids were placed in one group. All hemophilic Gram-negative bacilli were grouped as *B. influenzæ*, except the hemolytic type which was designated as Bacillus "X" (Pritchett and Stillman (20)).

RESULTS.

Graphic representation of the findings in the group studied is presented in Figs. 1 to 9. Brief discussions of each case accompany the charts. In the figures, the predominating organisms are shown by solid black circles and organisms with the second most numerous colonies, by blank circles; the presence of all other organisms is indicated by a plus sign.

Case 1 (K. C. M.), Fig. 1, was studied from October to June, inclusive. Two colds and one sore throat occurred during the period.

Normal Flora.—(1) *Nose: Staphylococcus albus*, present in 94 per cent of cultures, predominated. Diphtheroids were second most prominent (76 per cent). *Staphylococcus aureus* (30 per cent) occasionally predominated. Non-hemolytic streptococci and *Staphylococcus citreus* occurred once each. (2) *Throat:* Gram-negative cocci occurred in all cultures and usually predominated. Non-hemolytic streptococci occurred in all cultures, predominated a few times, but usually were

second in point of view of number of colonies. Bacillus "X" and diphtheroids were present in over half the cultures. Other bacteria were, "large Gram-positive coccus" (39 per cent), *Staphylococcus albus* (30 per cent), *citreus* (15 per cent), and *aureus* (12 per cent); pneumococci were found four times (12 per cent).

Colds.—The first cold (April) was moderately severe and was associated with a secondary bronchitis which required hospitalization. *Nose cultures* in the first 3

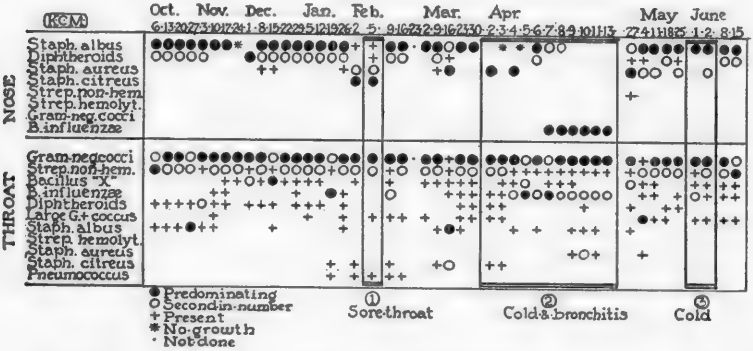

FIG. 1. Case 1. Graphic presentation of bacterial flora of nose and throat, normal, and during colds and throat infections.

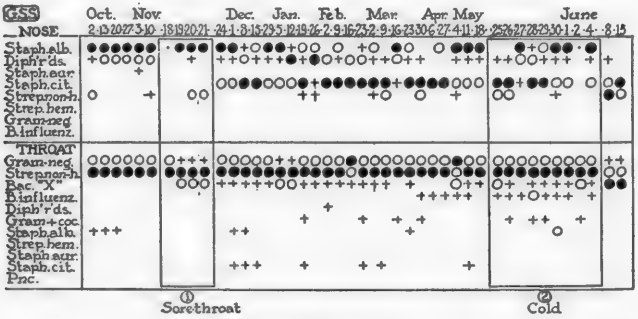

FIG. 2. Case 2.

days showed scanty growth with *Staphylococcus aureus* replacing all other organisms; on the 6th day *B. influenzæ* appeared and was present in pure culture on the 8th, 9th, 10th, and 11th days. *Throat cultures* showed little general change during the first 2 days. On the 3rd day *B. influenzæ*, which had been present before, became very prominent and remained so thereafter; at the same time the non-hemolytic streptococci decreased in numbers. *Staphylococcus aureus* appeared on

the 9th day and was very prominent on the 10th. Influenza bacilli were prominent in the sputum obtained upon coughing. No new or unusual organisms were noted in the early phases of the cold. The influenza bacilli, occurring at the period of secondary infection, may well be considered important secondary invaders.

The second cold (June) was of short duration. Aside from scanty growth in the nasal culture of the 1st day and in throat cultures of the 1st and 2nd days, and the prominence of *B. influenzæ* on the 1st day, no noteworthy change in the flora occurred. No new or unusual organisms were present.

Sore Throat.—This was mild and was associated with the presence of pneumococci in the throat cultures for 3 weeks preceding the onset.

Case 2 (G. S. S.), Fig. 2, was studied from October to June. One cold and one sore throat occurred during the period.

Normal Flora.—(1) *Nose: Staphylococcus citreus* (81 per cent) and *albus* (78 per cent) predominated about equally; diphtheroids (90 per cent) were next most

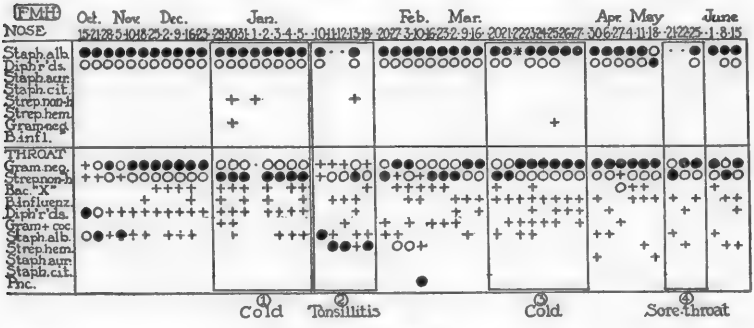

FIG. 3. Case 3.

prominent; non-hemolytic streptococci, long chains, were frequently present (32 per cent). Growth was usually very plentiful. (2) *Throat:* Non-hemolytic streptococci were present in all cultures and nearly always predominated. They frequently resembled pneumococci morphologically but were not bile-soluble nor pathogenic for white mice. Gram-negative cocci (100 per cent) were second most prominent. Bacillus "X" was common (68 per cent) and occasionally predominated. Less frequently noted organisms were, *Staphylococcus citreus* (25 per cent), *albus* (19 per cent), *B. influenzæ* (16 per cent), "Gram-positive cocci" (12 per cent), and diphtheroids (3 per cent).

Cold.—A moderately severe cold with a secondary bronchitis occurred in May. *Nose cultures* showed no variation from the normal flora. *Throat cultures* showed slight increase in prominence of *B. influenzæ*, which was present throughout, and prominence of *Staphylococcus albus* on the 6th day.

Sore Throat.—This was moderately severe and was accompanied by constitutional symptoms. It was associated with the first appearance of Bacillus "X" which was very prominent on the 2nd, 3rd, and 4th days. This was the first occurrence of the organism in the group. Its appearance in the others occasioned no untoward symptoms.

Case 3 (F. M. H.), Fig. 3, was studied from October to June. He had two colds, one attack of tonsillitis, and one sore throat.

Normal Flora.—(1) *Nose: Staphylococcus albus* occurred in all cultures and diphtheriods in 97 per cent; the former predominated every time but once. No other organisms appeared normally. The growth was usually very plentiful. (2) *Throat:* Gram-negative cocci and non-hemolytic streptococci occurred in all cultures. The former usually predominated. Other organisms were *Staphylococcus albus* (52 per cent), diphtheroids (52 per cent), Bacillus "X," and *B. influenzæ* (45 per cent), hemolytic streptococci (21 per cent), a little high, "large

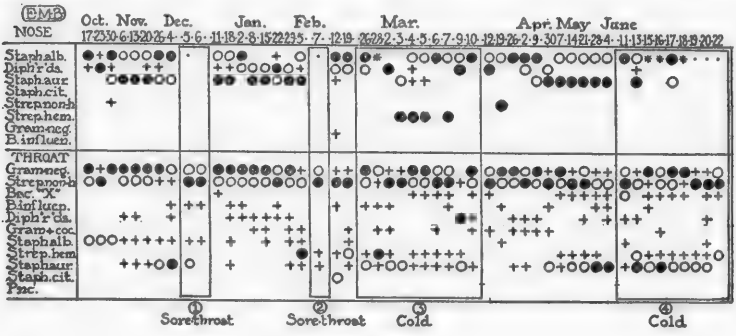

FIG. 4. Case 4.

Gram-positive cocci" (21 per cent), and *Staphylococcus aureus* (6 per cent). Pneumococci occurred and predominated once (Type IV) and were not accompanied by untoward manifestations.

Colds.—(1) This was a moderately severe cold (January) associated with a secondary bronchitis. *Nose cultures* showed scanty growth early; the general distribution was unaffected. Spread of Gram-negative cocci to the nose was noted once, of non-hemolytic streptococci twice. *Throat cultures*, Gram-negative cocci, usually predominating, were supplanted by non-hemolytic streptococci throughout. "Large Gram-positive cocci" made their first appearance on the 1st and 2nd days of the cold. No unusual organisms were noted.

(2) The second cold (March) was a typical one beginning in the throat. *Nose cultures:* Growth was scanty or absent early, Gram-negative cocci spread to the nose on the 6th day. *Throat cultures* showed no noteworthy changes. There were no new or unusual organisms present.

Tonsillitis.—This was a severe follicular tonsillitis requiring bed treatment. *Nose cultures:* No changes other than the spread of non-hemolytic streptococci on the 4th day. *Throat cultures:* The findings here were a little unusual. On the 1st day *Staphylococcus albus* was present in almost pure culture. On the 2nd day and thereafter hemolytic streptococci predominated. This sudden change was very striking.

Sore Throat.—This was very painful, showed acute pharyngeal inflammation and no constitutional symptoms. *Nose cultures:* No changes noted. *Throat cultures:* The variety of organisms was reduced and hemolytic streptococci were present on the 2nd day.

Case 4 (E.M.B.), Fig. 4, was studied from October to June. Two colds and two sore throats occurred during the period.

Normal Flora.—(1) *Nose: Staphylococcus aureus* (83 per cent) and *albus* (89 per cent) predominated about equally. Diphtheroids were frequent (66 per cent). Non-hemolytic streptococci and Gram-negative cocci appeared rarely. Growth was usually not very plentiful. (2) *Throat:* Gram-negative cocci, present in practically all cultures, predominated, while non-hemolytic streptococci (100 per cent) were second most prominent. *Staphylococcus aureus* was unusually frequent (58 per cent) and occasionally predominated. Other organisms present were *Staphylococcus albus* (58 per cent), diphtheroids (45 per cent), "large Gram-positive cocci" (38 per cent), *B. influenzæ* (31 per cent), Bacillus "X" (24 per cent), hemolytic streptococci (24 per cent), a little high, and *Staphylococcus citreus* (3 per cent).

Colds.—(1) A moderately severe cold (March) with sore throat which persisted on and off for a long time subsequently. *Nose cultures:* Growth was scanty at first and then increased. Hemolytic streptococci appeared and predominated on the 4th, 5th, 6th, and 8th days. This was an unusual finding. *Throat cultures:* The only noteworthy change was the prominence of both hemolytic streptococci and *Staphylococcus aureus*, organisms which had been present less frequently in preceding normal cultures. No new or unusual organisms were noted, at the onset.

(2) A moderately severe cold with a complicating laryngitis (June). *Nose:* The only change noted was scanty growth early. *Throat cultures:* Hemolytic streptococci increased; *Staphylococcus aureus* was very prominent but had been so in the normal cultures preceding the cold.

Sore Throats.—(1) Mild. Nose cultures not done. *Throat cultures: B. influenzæ* made its first appearance on the day preceding, was present during the sore throat, and was frequent in normal cultures thereafter.

(2) Mild. This was associated with the sudden first appearance of hemolytic streptococci, predominating, on the day preceding. This organism was frequently present in normal cultures after this.

Case 5 (D. W. A.), Fig. 5, was studied from October to June. He had no colds and no sore throats.

Normal Flora.—(1) *Nose: Staphylococcus albus* and diphtheroids were present in practically all cultures, the former usually predominating. *Staphylococcus aureus* was often present (45 per cent), and occasionally predominated. Non-hemolytic streptococci occurred occasionally. *Throat:* Gram-negative cocci, present in all cultures, strongly predominated. Non-hemolytic streptococci (100 per cent) were usually second in prominence. *B. influenzæ* was very frequently present (78 per cent) and occasionally predominated. Other organisms were

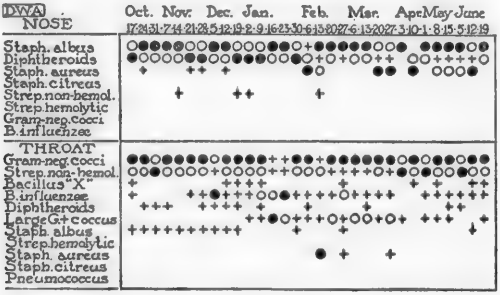

FIG. 5. Case 5.

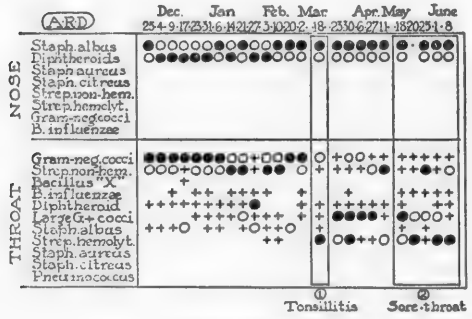

FIG. 6. Case 6.

"large Gram-positive cocci" (56 per cent), *Staphylococcus albus* (45 per cent), diphtheroids (38 per cent), Bacillus "X" (31 per cent), and *Staphylococcus aureus* (13 per cent).

Case 6 (A. R. D.), Fig. 6, was studied from December to June. He had one attack of tonsillitis and one chronic sore throat.

Normal Flora.—(1) *Nose: Staphylococcus albus* and diphtheroids were practically always present, the former usually predominating. No other organisms appeared. (2) *Throat:* Gram-negative cocci and non-hemolytic streptococci were always

present, the former predominated most often. "Large Gram-positive cocci" were very frequent (79 per cent) and occasionally predominated. Diphtheroids (84 per cent) and hemolytic streptococci (40 per cent) had a higher incidence than was usual in the group. Other organisms were *Staphylococcus albus* (58 per cent), *B. influenzæ* (47 per cent), and Bacillus "X" (5 per cent).

Tonsillitis.—Because of absence from the clinic, one culture only, at the end of the attack, was possible. The only noteworthy finding was the predominence of hemolytic streptococci in the throat culture.

Sore Throat.—This was a chronic inflammation of several weeks duration. *Nose cultures* showed no variation from the normal flora. *Throat cultures* showed hemolytic streptococci predominating with "large Gram-positive cocci" second most prominent. Gram-negative cocci and non-hemolytic streptococci were markedly reduced in numbers. *B. influenzæ* was a constant finding.

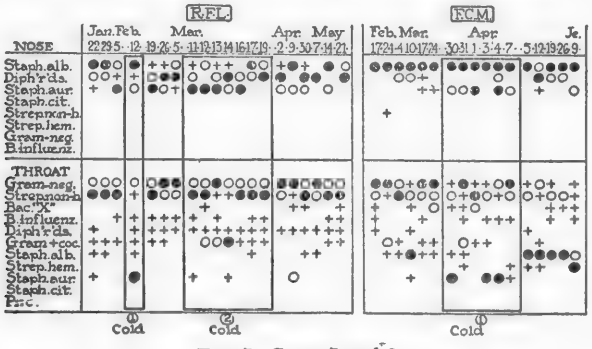

FIG. 7. Cases 7 and 8.

Case 7 (R. F. L.), Fig. 7, was studied from January to May. He had two colds.

Normal Flora.—(1) *Nose: Staphylococcus albus* (92 per cent), *aureus* (89 per cent), and diphtheroids (75 per cent) were the oganisms present. Diphtheroids predominated most often, then *albus* and *aureus* in order. Growth was usually very plentiful. (2) *Throat:* Non-hemolytic streptococci and Gram-negative cocci were nearly always present, the former usually predominating. Diphtheroids (89 per cent) were high. Other organisms were "large Gram-positive cocci" (58 per cent), *B. influenzæ* (50 per cent), *Staphylococcus albus* (33 per cent), *aureus* (17 per cent), and Bacillus "X" in 25 per cent of cultures.

Colds.—(1) The first (February) was a short uncomplicated cold. It was not reported early and only one late culture was obtained. *Nose culture* showed no unusual changes. *Throat cultures: Staphylococcus aureus* had become the predominating organism.

(2) A moderately severe cold with laryngitis (March). *Nose cultures: Staphylococcus aureus* became prominent from the very beginning. *Throat cultures:* "Large

Gram-positive cocci" became a little more prominent than usual on the 2nd and 4th days. No change in the general flora and no new or unusual organism noted.

Case 8 (F. C. M.), Fig. 7, was studied from February to June. She had one cold.

Normal Flora.—(1) *Nose: Staphylococcus albus* occurred in all cultures and markedly predominated. Diphtheroids were second most prominent. Other

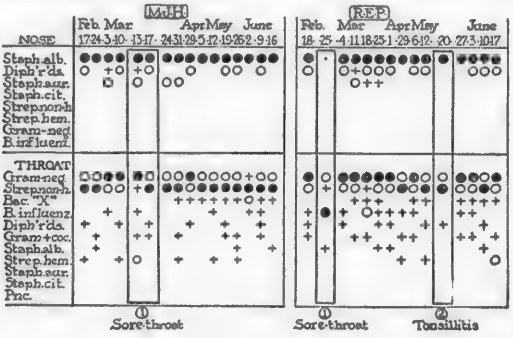

FIG. 8. Cases 10 and 11.

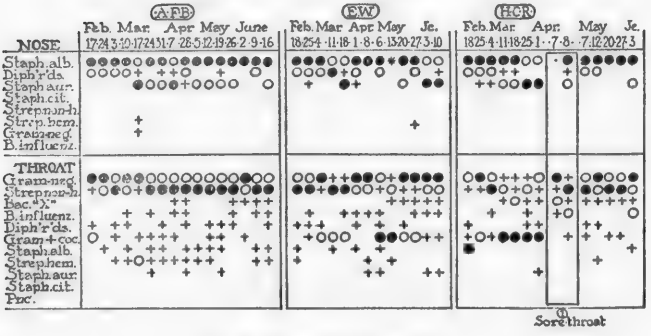

FIG. 9. Cases 9, 12, and 13.

organisms were *Staphylococcus aureus* (34 per cent), and non-hemolytic streptococci (9 per cent). (2) *Throat:* This was unusual. *Staphylococcus albus* was always present, and usually predominated. Gram-negative cocci (91 per cent) were second most prominent and non-hemolytic streptococci were always present. Other organisms were Bacillus "X" (55 per cent), "large Gram-positive cocci' (44 per cent), *B. influenzæ* (34 per cent), hemolytic streptococci (27 per cent), high, diphtheroids (27 per cent), and *Staphylococcus aureus* (9 per cent).

Cold.—This (April) was severe and was complicated by bronchitis and sinusitis. *Nose cultures: Staphylococcus aureus* became unusually prominent and remained so throughout. *Throat cultures: Staphylococcus aureus* was prominent from the beginning; *albus* was less conspicuous than usual.

TABLE I.

Percentage Incidence of Bacteria in the Noses and Throats of Normals.

Cases.	Total cultures (weekly).	Nose. Organisms.							Throat. Organisms.										
		Staphylococcus albus.	Diphtheroids.	*Staphylococcus aureus.*	*Staphylococcus citreus.*	Streptococcus non-hemolytic.	Gram-negative cocci.	Streptococcus hemolytic.	Gram-negative cocci.	Streptococcus non-hemolytic.	Bacillus "X."	*B. influenzæ.*	Diphtheroids.	"Large Gram-positive cocci."	*Staphylococcus albus.*	Streptococcus hemolytic.	*Staphylococcus aureus.*	*Staphylococcus citreus.*	Pneumococci.
1. K.C.M.	33	94	76	30	3	3			100	100	64	42	60	39	30		12	15	12
2. G.S.S.	31	78	90	3	81	32			100	100	68	16	3	10	19			25	
3. F.M.H.	29	100	97						100	100	45	45	52	21	52	21	6		3
4. E.M.B.	29	87	67	83		7	3		97	100	24	31	45	38	48	24	58	3	
5. D.W.A.	32	97	97	45		12			100	100	31	78	38	56	45		13		
6. A.R.D.	19	100	95						95	100	5	47	84	79	58	40			
7. R.F.L.	12	92	75	89					100	100	25	50	89	58	33		17		
8. F.C.M.	11	100	55	34		9			91	100	55	34	27	44	91	27	9		
9. A.F.B.	16	100	63	75			6	6	100	100	38	44	56	56	63	69	19		
10. M.J.H.	14	100	50	21					100	100	57	29	35	35	21	35			
11. R.E.P.	13	100	84	23					100	100	69	69	46	61	23	23			
12. H.C.R.	12	100	58	58					100	100	75	25	42	83	17	8	8		
13. E.W.	14	93	71	50					100	100	36	64	43	78	29	21	29		
Average, *per cent*.....		92	79	36	10	7	1	0.4	99	99	49	47	45	45	40	17	14	5	2

Cases 9 to 13, Figs. 8 and 9, were studied from February to June. They developed no colds. Three had sore throats.

Normal Flora.—(1) *Nose:* In all cases *Staphylococcus albus* predominated. All had diphtheroids and *Staphylococcus aureus*, three showing the former second most prominent, and two the latter, Gram-negative cocci appeared in two, and one showed a hemolytic streptococcus once. (2) *Throat:* Three had non-hemolytic streptococci predominating and Gram-negative cocci second. This order was reversed for the other two. Hemolytic streptococci were high in two, A. F. B., 69 per cent, and M. J. H., 35 per cent. Other findings were essentially similar to the previous cases discussed.

Sore Throats.—These were four in number, of varying severity. Hemolytic streptococci were prominent in one of these (M. J. H.). Otherwise no noteworthy departures from normal findings were observed.

Summary of Results.

Normal Cultures.—A summary of the percentage incidence of all organisms obtained in cultures from the noses and throats of normal individuals is detailed in Table I. The total number of cultures per person appears in the first column; both aerobic and anaerobic cultures are combined as one. The organisms are arranged in order of greatest average occurrence.

In the consideration that follows, an attempt is made to divide the bacteria into those that are practically always present, essential or basic normal flora; and those that are frequently or occasionally present, temporary or transient flora. Consideration is given also to the presence of organisms to which pathogenic rôles are often attributed.

Nose.—The essential normal flora includes *Staphylococcus albus*, occurring in 92 per cent of cultures, and diphtheroids, 76 per cent. The former nearly always predominated. This is in agreement with Bloomfield's findings (21). In some individuals, however, certain other organisms were practically as frequent. In Cases 4, 7, and 9, *Staphylococcus aureus* was present in 75 to 89 per cent, and in Case 2, *Staphylococcus citreus* was found in 81 per cent. For these individuals these organisms may well be accorded the place of basic normal flora. A less likely explanation would be that these constitute an exaggerated carrier state, although no apparent foci of infection were present. Transients included non-hemolytic streptococci occasionally (high in Case 2, 32 per cent) and rarely, Gram-negative cocci and hemolytic streptococci.

So called potential pathogens were found in normal cultures only once, hemolytic streptococci in Case 9. There were no associated untoward symptoms.

Throat Cultures.—The basic flora includes Gram-negative cocci and non-hemolytic streptococci which appeared in practically all cultures. The former predominated in 55 per cent and the latter in 33 per cent of the cultures. This is in accord with Bloomfield's

findings (16). As in the case of the nose cultures, certain other organisms are unusually prominent in some individuals, again perhaps assuming a place among the essential flora. *B. influenzæ*, for example, was present in 64 to 78 per cent of cultures from Cases 5, 11, and 13; Bacillus "X" in 55 to 75 per cent, in Cases 1, 2, 8, 10, 11, and 12. Diphtheroids ranged from 56 to 89 per cent in Cases 1, 6, 7, and 9; and "large Gram-positive cocci" were present in 56 to 83 per cent, in Cases 5, 6, 7, 9, 11, 12, and 13. One hesitates a little in including these organisms under the head of general basic flora; and yet, for certain individuals this assignation seems correct, although for others the organisms belong to the class of frequent transients.

Intermediate between basic and transient flora comes *Staphylococcus albus* with an average incidence of 40 per cent. True transients include hemolytic streptococci, *Staphylococcus aureus* and *citreus*, and pneumococci.

Bacteria occasionally assumed to play pathogenic rôles had a high incidence in some of the cases. Hemolytic streptococci were very frequent in Case 9 who had neither colds nor sore throats, and in Case 8 where there was little apparent effect. Incidence was high in Cases 4 and 6 who had more or less continuous subacute inflammation of their throats. The organism was prominent in the colds of the former. *Staphylococcus aureus* was common in Case 4, and the organism was somewhat more prominent in her colds. Pneumococci were rare. In Case 1, their presence was associated with a mild sore throat 3 weeks after their initial appearance. In Case 3, their occurrence resulted in no untoward symptoms. High incidence of *B. influenzæ* and of the hemolytic type, Bacillus "X," was not associated with any apparent increase in respiratory infection.

Cultures during Colds.—In studying the bacterial flora of colds an attempt has been made to determine (1) whether any new or unusual organisms are present to which an etiological rôle may be assigned, (2) the relationship, if any, to colds, of transient, so called potential, pathogens, and (3) the qualitative and quantitative changes in the usual flora in the course of colds.

In answer to the first question it may be stated that no bacteria were found in either the first or early cultures to which a causative rôle could be attributed. In Case 3 only, did an organism appear

for the first time in a first cold culture. This was a "large Gram-positive coccus" to which only slight significance can be assigned in view of its frequent occurrence in the usual essential flora. It is worthy of note, however, that its incidence in this individual's normal cultures was comparatively low (21 per cent).

Among the organisms to which a pathogenic rôle has been variously assigned, the following were noted, hemolytic streptococci, *Staphylococcus aureus*, and pneumococci. For purposes of discussion we may include also the influenza bacilli, both non-hemolytic and hemolytic (Bacillus "X").

TABLE II.

Average Percentage Incidence of Bacteria in Cultures from Normals, Colds, and Sore Throats.

	Nose.								Throat.									
	Staphylococcus albus.	Diphtheroids.	*Staphylococcus aureus.*	*Staphylococcus citreus.*	Streptococcus non-hemolytic.	Gram-negative cocci.	Streptococci hemolytic.	*B. influenzæ.*	Gram-negative cocci.	Streptococcus non-hemolytic.	Bacillus "X."	*B. influenzæ.*	Diphtheroids.	Gram-positive cocci.	*Staphylococcus albus.*	Streptococcus hemolytic.	*Staphylococcus aureus.*	*Staphylococcus citreus.*	Pneumococci.
Normal.....	92	79	36	10	7	1	0.4	—	99	99	49	47	45	45	40	17	14	5	2
Colds.....	73	52	28	12	8	3	6	9	99	99	57	67	39	43	27	26	40	4	—
Sore throats.	100	77	35	12	6	—	—	—	100	100	26	70	35	48	48	56	9	—	9

Table II shows a comparison of the percentage incidence of all organisms present in normal cultures, and those obtained during colds. It is interesting to note that practically all the potential pathogens enumerated above show an increase in either the nose or throat cultures.

Nose Cultures.—*Staphylococcus aureus* shows a decrease, paralleling *Staphylococcus albus* (normal basic flora). It should be noted that it was unduly prominent in the first cold of Case 1. *B. influenzæ* and hemolytic streptococci show increases, which, however, are due chiefly to late secondary spread in Cases 1 and 4 respectively.

Throat Cultures.—Staphylococcus aureus shows considerable increase. This, however, is in part apparent as Case 4, with a high normal incidence and a correspondingly high incidence in her colds, is responsible for most of the raised average. This organism was prominent in two other colds; early in one and late in another. The increase of hemolytic streptococci was slight and constitutes secondary invasion. The occurrence of *B. influenzæ* was definitely high during colds. This increase also is more or less apparent as part is due to the prominence of the organism in the secondary phases of

TABLE III.

Percentage of Predominance of Various Bacteria in Cultures from Normals and from Colds.

	Nose.								Throat.									
	Staphylococcus albus.	Diphtheroids.	*Staphylococcus aureus.*	*Staphylococcus citreus.*	Streptococcus non-hemolytic.	Gram-negative cocci.	Hemolytic streptococcus.	*B. influenzæ.*	Gram-negative cocci.	Streptococcus non-hemolytic.	Bacillus "X."	*B. influenzæ.*	Diphtheroids.	Gram-positive cocci.	*Staphylococcus albus.*	Streptococcus hemolytic.	*Staphylococcus aureus.*	*Staphylococcus citreus.*	Pneumococci.
Normal.....	70	13	10	6	1	—	—	—	55	33	1	1	1	4	4	1	2	—	0.5
Colds.....	57	6	15	6	—	—	6	10	39	46	—	3	1	1	—	1	9	—	—

some of the colds, and part may be attributed to its uniformly high incidence in normal as well as cold cultures at the time of occurrence of most of the colds (late winter and spring). The moderate rise of Bacillus "X" is similarly due to its more widespread presence at the same time. Pneumococci played no part in the colds of the series.

Quantitative and qualitative changes occurred in the general and basic nature of the flora in most of the colds. In early cultures from the nose, scanty or even absent growth was moderately conspicuous. Such quantitative changes were not so noticeable in throat material. Qualitative changes were noticeable in both. Reference to Table II shows that the average incidence of the nasal normal basic flora, *i.e. Staphylococcus albus* and *aureus*, and diphtheroids, is decreased;

and the individual charts of cases show late increase in variety resulting from secondary spread.

In the throat cultures the incidence of basic organisms is unaffected but changes in their predominance are noticeable. Table III brings these out. From this it will be observed that the usually more prominent Gram-negative cocci fall off and that their conspicuous place is taken by non-hemolytic streptococci. On inspection of the cold plates, this was particularly striking. *Staphylococcus aureus* also rises a little in prominence. Another noteworthy change was the general tendency to an increase in the variety of organisms in later throat cultures.

Sore Throats.—The primary concern of this study was with colds. However, cultures were made in the course of tonsillitis and sore throats. The results are shown in the individual charts and in Table II. The positive findings were, in brief, no change in nasal cultures and the more or less expected striking increase, in throat cultures, of hemolytic streptococci, from 17 per cent in normals to 56 per cent in throat infections. The high incidence of *B. influenzæ* may be accounted for on the same grounds as noted for colds. The possible relationship of pneumococci to throat infection in Case 1, and of Bacillus "X" in Case 2 has been referred to above.

SUMMARY AND CONCLUSIONS.

1. The normal bacterial flora of the nose and throat of thirteen individuals has been studied over periods ranging from 5 to 9 months.

2. Observations have been made of qualitative and quantitative changes in the flora occurring in the course of colds and of throat infections appearing in the group.

3. The normal basic nasal flora includes *Staphylococcus albus*, diphtheroids, and for certain individuals *Staphylococcus aureus* and *citreus*. Occasional transient bacteria are Gram-negative cocci and non-hemolytic streptococci.

4. The normal basic throat flora includes Gram-negative cocci, non-hemolytic streptococci, and for certain individuals "large Gram-positive cocci," *B. influenzæ*, Bacillus "X," and diphtheroids. Transient organisms are *Staphylococcus albus*, hemolytic streptococci. *Staphylococcus aureus* and *citreus*, and pneumococci.

5. No bacteria were found in early cold cultures to which a causative rôle could be assigned.

6. In the course of colds the basic flora of the nose was often scanty in the early stages. The throat showed reduction of prominence or alterations in predominance of the basic flora.

7. Certain organisms were prominent in colds, usually as late or secondary invaders, these included *Staphylococcus aureus*, hemolytic streptococci, and *B. influenzæ*.

8. There was a striking increase in the incidence of hemolytic streptococci in throat infections.

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ON THE FREQUENCY OF SPONTANEOUS TUMORS IN THE DOMESTIC FOWL.

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INTRODUCTION.

Several cases of the occurrence of fowl tumors in apparently epidemic form have led to an inquiry regarding the normal frequency of tumors in the domestic fowl. The only published data on this subject which have come to our attention are two papers, one by Curtis (1915–16) in which the results of autopsies of 880 birds are summarized, and one by Ehrenreich (1907) based upon examination of 4000 fowls which had been killed for eating purposes. In both these cases the specimens varied in age. We are now able to add to these data the results of autopsies of all birds dying in a population of some 11,000 individuals, all members of a single age group and comparable with regard to methods of treatment.

Source of Data.

The conclusions and table in this paper are based upon data from three main sources, the Storrs International Egg Laying Contest held at this station, the Harper Adams Egg Laying Trials conducted by the Harper Adams Agricultural Collegé at Bentley, Salop., England, and farm flock records supplied by Dr. H. D. Goodale, to whom grateful acknowledgement is made. The data from both contests are based upon records of the past 4 years, 1920 through 1924; those from the farm flock upon a single year's records, 1923–24. All the birds involved were pullets and they comprise a homogeneous group with regard to age, being approximately 6 to 18 months old during the period in which they were under observation.

A thousand birds were sent each year to the Storrs Contest and these came, during the time with which we are dealing, from twenty-three states in this country and from England, British Columbia, and Ontario, and include eleven breeds of fowl. They probably represent a random sample of birds bred for egg produc-

* Paper 38.

tion in this country. The birds in the Harper Adams Trials have varied in number from year to year as given in the table. They were of various breeds and may be considered representative of English farm flocks. The data supplied by Goodale are based upon about 1300 birds, for the most part White Leghorns but including a few Buff Wyandottes and Rhode Island Reds.

Gross autopsies of all birds which died were carefully made. Those of the Storrs Contest birds were made by Mr. H. T. Covell, Superintendent of the Contest, who recorded cause of death, when determined, and any abnormalities found. Those of the Harper Adams birds were conducted by the Ministry of Agriculture and the findings published in the December numbers of the *Utility Poultry Journal*. The farm flock autopsies were made by Dr. Goodale. The examinations were, in most cases, gross, and only the more obvious lesions were observed. In this paper, however, we are chiefly interested in determining the frequency of tumors on macroscopic examination and for this limited purpose the data are sufficient. No distinction is drawn between malignant and benign growths nor does the fact that a bird which died had a tumor necessarily imply that the tumor was the cause of death. Conversely, the fact that a bird did not die does not indicate that it was not actually or potentially a tumor bearer. These facts must be borne in mind when dealing with the data.

Comparability of Data.

The treatment of the birds in the contests has been uniform throughout with one exception to be noted later. They have been sent to the contests on November 1, following the spring in which they were hatched, at an age of 6 or 7 months and have been under observation for the ensuing year through October 31. The population of the contests is naturally heterogeneous while that of the farm flock is homogeneous in a broad sense. The birds in all groups receive optimum care. Autopsy methods are comparable and, though there may be some differences in the diagnosis of tumor, the error due to difference in observers is probably slight.

In the last 2 years, beginning November, 1922, and November, 1923, the birds in the Storrs Contest have been subjected to methods for forcing egg production. From the first of November through March electric lights were turned on from 9 to 10 in the evening and the birds given an extra feed in the evening. This procedure was followed again from the first of September through October. Along with the use of lights, the protein content of the feed was increased. The result of these forcing measures has been a relative increase in the average egg production during the winter and fall months and a relative decrease during the spring and summer months.

Description of Table.

The accompanying table has been constructed to show the annual and seasonal variation of tumor frequency in the different flocks. As

TABLE I.

Summary of the Occurrence of Tumors in Fowls.

Date.	Source of data.	No. of birds.*	No. which died.	No. with tumors.	Per cent of dead with tumors.	Per cent mortality.	Per cent of total population with tumors.
1920–21							
Nov.–Apr.	Storrs Contest.	986	52	8	5.4	5.3	0.81
May–Oct.	" "	920	78	15	19.2	8.5	1.63
Total.	" "	**972**	**130**	**23**	**17.7**	**13.4**	**2.37**
1921–22							
Nov.–Apr.	" "	991	52	5	9.6	5.2	0.50
May-Oct.	" "	921	97	16	16.5	10.5	1.74
Total.	" "	**973**	**149**	**21**	**14.1**	**15.3**	**2.16**
1922–23†							
Nov.–Apr.	" "	975	94	7	7.4	9.6	0.72
May–Oct.	" "	868	84	8	9.5	9.7	0.92
Total.	" "	**962**	**178**	**15**	**8.4**	**18.5**	**1.56**
1923–24†							
Nov.–Apr.	" "	983	84	16	19.0	8.5	1.63
May–Oct.	" "	863	77	11	14.3	8.9	1.27
Total.	" "	**947**	**161**	**27**	**16.8**	**17.0**	**2.85**
1920–24							
Nov.–Apr.	" "	3935	282	36	12.8	7.2	0.91
May–Oct.	" "	3572	336	50	14.9	9.4	1.40
Total.	" "	**3854**	**618**	**86**	**13.9**	**16.0**	**2.23**
1920–21							
Nov.–Apr.	Harper Adams Contest.	695	16	0	0.0	2.3	0.00
May–Oct.	" " "	675	30	3	10.0	4.4	0.44
Total.	" " "	**691**	**46**	**3**	**6.5**	**6.7**	**0.43**
1921–22							
Nov.–Apr.	" " "	1747	79	3	3.8	4.5	0.17
May–Oct.	" " "	1649	59	4	6.8	3.6	0.24
Total.	" " "	**1728**	**138**	**7**	**5.1**	**8.0**	**0.41**
1922–23							
Nov.–Apr.	" " "	1950	38	6	15.8	2.0	0.31
May–Oct.	" " "	1909	73	15	20.5	3.8	0.79
Total.	" " "	**1947**	**111**	**21**	**18.9**	**5.7**	**1.08**
1923–24							
Nov.–Apr.	" " "	1952	42	9	21.4	2.2	0.46
May–Oct.	" " "	1904	65	12	18.5	3.4	0.63
Total.	" " "	**1946**	**107**	**21**	**19.6**	**5.5**	**1.08**

* Excluding those which died from accident or from contagious disease.

† Electric lights were used during the winter and fall months. See text.

TABLE I—*Concluded.*

Date.	Source of data.	No. of birds.*	No. which died.	No. with tumors.	Per cent of dead with tumors.	Per cent mortality.	Per cent of total population with tumors.
1920–24							
Nov.–Apr.	Harper Adams Contest.	6344	175	18	10.3	2.8	0.28
May–Oct.	" " "	6137	227	34	15.0	3.7	0.55
Total.	" " "	**6312**	**402**	**52**	**12.9**	**6.4**	**0.82**
1923–24							
Nov.–Apr.	Farm flock.	1294	273	89	32.6	21.1	6.88
May–Oct.	" "	975	389	71	18.3	39.9	7.28
Total.	" "	**1240**	**662**	**160**	**24.2**	**53.4**	**12.90**

indicated in the column at the left, the contest year has been divided for our purposes into two periods of 6 months each, November 1 through April 30 and May 1 through October 31. The second column gives the source of the data; the third the total number of birds under observation upon which the percentages are based. This is taken as the total number of birds submitted to the contest less the number which died during the particular period concerned from contagious disease, less (in the case of the May through October period) the number which died during the first period. In the fourth column are listed the numbers which died from all causes exclusive of contagious disease. The fifth column gives the number of birds which, upon autopsy, showed tumors and the sixth the per cent with tumors of the total dead. In the next column is given the per cent mortality and in the last the per cent with tumors. This last figure is based upon the total population and includes only the birds with tumors which died. It does not take into account those which were developing tumors at the end of their pullet year or were hosts of benign tumors which did not materially affect the health of the birds. The annual totals and percentages are given in each case and are obtained in the same general way as the seasonal totals.

Data from Other Sources.

Curtis (1915–16) obtained an estimate of tumor rate in fowls bred at the Maine Experiment Station based upon 660 birds which died and 220 apparently normal

birds which were killed. The two groups did not differ appreciably in the per cent of tumors found and when these were grouped, a tumor rate of 8.98 per cent was obtained. This was based upon birds of various ages, including 836 females and 44 males. When these were divided into age groups including fowls dying at from ¼ to 2¼ years of age, and at 2¼ years and over, a distinct rise in the tumor rate with increased age is demonstrated, the younger group having a rate of 7.37 per cent (based upon 760 individuals) as compared with 19.17 per cent (based upon 120 individuals) for the older group. This is in accordance with the condition known to exist in many mammals in which there is a high degree of correlation between the occurrence of tumor and increase in age.

Ehrenreich (1906, 1907) examined 4000 birds which were brought into a hotel for eating purposes and found 7 malignant tumors, all occurring in 1000 hens over 1 year old.

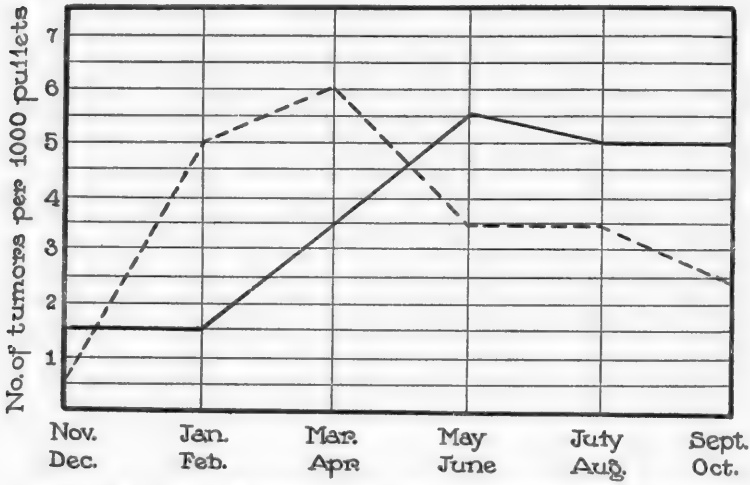
Lentz (1922–23) has given data on the causes of death in poultry at the Massachusetts Agricultural Experiment Station. In 8 out of 297 deaths (deaths due to contagious disease are omitted) tumors were involved and these were scattered through a flock of about 1500 fowls, giving a tumor rate of about one-half of 1 per cent based upon the total population.

In addition to the published data, records of about 500 pullets hatched and reared at this station have been examined and a tumor rate of 3.24 per cent obtained.

DISCUSSION.

We may now look at the data as summarized in the table. In the last column it will be observed that, with one exception, the second half year has a consistently higher tumor rate than the first. The differences are slight, in most cases less than 1 per cent, but they vary constantly in one direction and are probably significant. Just what factors are involved in the seasonal difference is by no means certain. Age is known to be a factor in mammals and Curtis (1915–16) has supplied evidence that it is also one in birds. That the factor of age, however, is operative within such a relatively short period is extremely doubtful and Curtis's data gave no such indication. It is conceivable that some causal relation exists between tumor frequency and antecedent egg production since about half of the total number of tumors are associated with the ovary or oviduct. The inference is that reproductive overwork in the spring results in conditions favorable to the growth of spontaneous tumors. The peak of egg production comes in April and May so that if overfunctioning

of the reproductive system is a factor influencing the tumor rate, its effect should be noticeable after that time; that is, in the second or May through October period. By this same hypothesis of the causal relation between excessive egg production and tumor frequency the reversed condition in the year of the Storrs Contest beginning 1923 may be explained. In this year the tumor rate for the first 6 months is



TEXT-FIG. 1. Curves showing relation between increased egg production and tumor frequency.

——— 2 years beginning November, 1920 and 1921. No lights used. Egg production normal. Peak of tumor frequency comes in early summer.

- - - - - - 2 years beginning November, 1922 and 1923. Lights and feed with higher protein content used. Winter egg production increased. Peak of tumor frequency comes in early spring.

1.63 per cent as compared with 1.27 per cent for the second. In this and the previous year, winter egg production was forced by the use of lights and feed with a high protein content so that, although a larger number of eggs was still laid in the spring, the greatest strain upon the reproductive organs was sustained in the winter months since the deviation from the normal was greatest at that time. The winter production was higher and the following spring production lower than the average. The use of forcing measures has apparently a

levelling effect. On the assumption that an increase in tumor rate follows reproductive overwork, we should expect a relative increase of tumors in the first half year and a decrease in the second, for under these abnormal conditions the strain comes earlier in the year and the resultant increase in tumor rate is shifted ahead so that most of it, but probably not all, is transferred into the November through April period. Some of the effect probably lags into May. In the year 1922–23, during which measures for forcing egg production were also used, the tumor percentages were very nearly equal in the two half year periods.

The effect of lights and high protein food is graphically shown in the figure. The unbroken line represents the number of tumors for each 2 month period for the 2 years beginning November, 1920, and November, 1921, and before forcing measures were used. The broken line represents tumor frequency for the years beginning November, 1922, and November, 1923, when lights were used. Under normal farm conditions it will be seen that the tumor frequency reaches its peak in May and June (unbroken line) and maintains approximately the same level throughout the summer and fall following. This may be regarded as the normal distribution of tumors through the pullet year. When methods for forcing egg production are used, however, the peak of tumor frequency may come 2 or 3 months earlier, and may show a distinct decrease in May and June and subsequently a slight but probably not significant decline. The interpretation is that the spring rise in tumor rate is definitely associated with increased egg production.

One phenomenon which has come to our attention not infrequently is the appearance of tumors in what seems to be epidemic form. In one flock about 30 out of 100 pullets which died showed tumors on gross examination, a tumor rate of about 10 per cent based on the whole population of 300 pullets. This increased tumor rate appeared suddenly. In the year previous to the one in question the frequency had been normal. Such sporadic cases, however, are probably to be considered separately from the normal tumor rate and suggest that these "epidemic" forms differ in etiology from those making up what may be considered the normal rate. Such an explanation may be

applied to the farm flock cited in the table for obviously there is a significant difference between the rate of 12.9 per cent in the farm flock and those of 2.23 and 0.82 per cent as found for the two contests.

The difference between the Storrs rate (2.23 per cent) and the Harper Adams rate (0.82 per cent) is significant and is probably due to the higher mortality rate existing in the former. In her work, Curtis found no sensible difference in tumor frequency between two groups, one of which consisted of 660 birds which died and the other of 220 apparently normal birds which were killed. The former had a tumor rate of 8.94 per cent as compared with 9.09 per cent for the latter. In the first group not more than half the deaths were due primarily to tumor. Although this seems to be rather an exaggerated case, it does indicate that more tumors will be found when the mortality rate is high since tumors frequently are not the primary cause of death. Since the mortality rate of the Storrs Contest has been higher throughout than that of the Harper Adams, one would expect to find a larger percentage of tumors, but on the other hand approximately the same relationship should hold between the number of tumors found and the number of birds which die. It may be seen from the table (Column 6) that the percentage of the dead showing tumors is practically the same, 13.9 per cent of the birds dying in the Storrs Contest showing tumors and 12.9 per cent of the Harper Adams birds which died showing tumors. Obviously then, wherever a larger number of birds die, from whatever cause, more tumors will be found. There is probably no real difference in the number of spontaneous tumors occurring in the two flocks, and the normal tumor rate for female fowls between 6 and 18 months of age may be given as approximately 2 or 3 per cent.

SUMMARY.

1. Analysis of records from the Storrs Contest, the Harper Adams Trials, and a farm flock indicate an increase of tumors in the second half of the pullet year (May through October) over the first half (November through April).

2. Some evidence seems to indicate that heavy antecedent egg production is associated with a rise in tumor rate.

3. The normal annual tumor rate for fowls between the ages of 6 and 18 months is about 2 or 3 per cent.

4. The appearance of tumors in "epidemic" form probably indicates that the neoplasms found differ in etiology from those upon which the normal rate is based.

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